Dissecting the role of iASPP, a novel crucial regulator of epidermal homeostasis, in squamous cell carcinoma

Deborah Robinson

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Centre for Cell Biology and Cutaneous Research, Blizard Institute
Barts and The London School of Medicine & Dentistry
Queen Mary, University of London

Supervisors: Dr Daniele Bergamaschi
Professor Catherine Harwood

Statement of originality

I, Deborah Robinson, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated. Previously published material is also acknowledged below.

I attest that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge break any UK law, infringe any third party's copyright or other Intellectual Property Right, or contain any confidential material.

I accept that the College has the right to use plagiarism detection software to check the electronic version of the thesis.

I confirm that this thesis has not been previously submitted for the award of a degree by this or any other university.

Signature: Deborah Robinson

Date: 11/01/16

Publications and presentations

Publications arising from this thesis

Robinson DJ, Patel A, Rizvi H, Chiorino G, Harwood CA & Bergamaschi D. *The role of the iASPP-p63 axis in cutaneous squamous cell carcinoma*. Manuscript in preparation.

Publications I have contributed to

Akinduro O, Sully K, Patel A, Robinson DJ, Chikh A, McPhail G, Braun K, Philpott MP, Harwood CA, Byrne C, O'Shaughnessy RFL & Bergamaschi D. *Constitutive autophagy during epidermal development.* Manuscript in preparation.

Presentations arising from this thesis

Oral Presentation at the British Society for Investigative Dermatology (BSID) annual meeting (Southampton, UK; April 2015). **Best Oral Presentation - Runner Up.**
Dissecting the role of iASPP, a novel regulator of epidermal homeostasis, in keratinocyte skin carcinogenesis

Oral Presentation (and poster) at the Blizard Graduate Studies Day, Barts and The London (London, UK; April 2015).
Dissecting the role of iASPP, a novel regulator of epidermal homeostasis, in keratinocyte skin carcinogenesis

Oral (and poster) presentation at the European Society for Dermatological Research (ESDR) annual meeting (Copenhagen, Denmark; September 2014).
Dissecting the role of iASPP, a novel regulator of epidermal homeostasis, in keratinocyte skin carcinogenesis

Poster Presentation at William Harvey Day, QMUL (London, UK; October 2013).
Dissecting the role of iASPP, a novel regulator of epidermal homeostasis, in keratinocyte skin carcinogenesis.

Abstract

Previous data have unveiled a novel autoregulatory feedback loop between iASPP and p63 in the stratified epithelia; this involves two microRNAs, miR-574-3p and miR-720, and is critical for epidermal homeostasis. The iASPP oncoprotein, an inhibitory member of the ASPP (apoptosis stimulating protein of p53) family, is a key inhibitor of p53 and NF-κB and is highly expressed in many cancers. Non-melanoma skin cancer, comprising of cutaneous squamous carcinoma (cSCC) and basal cell carcinoma, is currently the most common malignancy in the UK. In view of this newly-identified iASPP-p63 axis, I hypothesised a potential role for dysregulation of this feedback loop in the pathogenesis of cSCC and aimed to assess the role of iASPP in human cSCC.

Protein and mRNA expression patterns were assessed in a panel of 10 cSCC cell lines generated by our group. In addition, immunostaining of iASPP and p63 was performed in 107 cSCC clinical samples of variable differentiation status. The data reveal an overall increase in expression of iASPP and ΔNp63 in cSCC but also suggest a significant alteration of the cellular localisation of iASPP dependent on the differentiation status of the tumour. To further assess the effects mediated by the iASPP/p63 axis, iASPP and p63 have been silenced by RNAi technology in a subset of cSCC cell lines. Whilst data shows the direct effects of iASPP and p63 upon each other's expression are maintained in cSCC, epigenetic dysregulation of the feedback loop at the microRNA level may be occurring via a novel p63 regulator, miR-211-5p. Functionality of iASPP in cSCC (proliferation, apoptosis, cell motility/migration and invasiveness) provides evidence for a role of iASPP in preventing epithelial-mesenchymal transition in cSCC via a p63/miR-205-5.

These findings provide potential future directions for development of clinical biomarkers and novel therapeutic targets for cSCC and may ultimately provide the tools for tackling the increasing morbidity and mortality associated with this malignancy.

Acknowledgements

First and foremost, I would like to thank my two supervisors Dr Daniele Bergamaschi and Professor Catherine Harwood. I am extremely grateful to them both for welcoming me into their research group and for their continuous support throughout my time here. I would like to thank Daniele for always being available for advice and stopping me from needlessly panicking/worrying and I would like to thank Catherine for her never ending enthusiasm and support, despite the numerous other projects she has going on. I am also very thankful to the British Skin Foundation for funding me throughout my PhD and to all the patients who agreed to participate in this research.

I would like to say special thanks to Dr Hasan Rizvi and the Core Pathology department for all their wisdom and assistance in staining and scoring my patient tissues. I am very grateful to past and present members of the lab, in particular Dr Anissa Chikh and Paolo Sanza for guiding me in my early days and teaching me many experimental techniques used in this thesis. I am truly thankful to Ankit Patel for his continued willingness to help with microRNA techniques and experiments and I am very grateful to Dr Karin Purdie for all her help with cell lines and patient tissues. I would also like to thank our collaborators in Cologne University, Dr Baki Akgül and Dr Martin Hufbauer for carrying out the *in situ* hybridisation experiments in this thesis and Dr Giovanna Chiorino from Fondazione Edo ed Elvo Tempia for carrying out the microRNA array and analysis. I am also very appreciative for the statistical help Dr Ai Nagano provided.

I owe thanks to members of the Blizard institute including Dr Matthew Caley for his guidance with my 3D Organotypic cultures, Dr Adrian Biddle and Dr Luke Gammon for their EMT advice. In addition, Emma, Ngoc, Kasia, Anke and Hannah, thank you for all the scientific (and non-scientific!) discussions we have had over the past three years. I would like to thank my family, in particular my Mum and Dad for supporting and encouraging me in all my education so far. Also I would like to say a big thank you to my friends for their continued concern into 'how my cells are doing'. Lastly I would like to thank my partner Oli for his continued ability to see the bright side of every event in life and for all the time he has spent helping me practise for various presentations, though I know that really you were just watching football on your phone all along.

Contents

List of figures

List of tables

Abbreviations

Abbreviation	Definition
AK	Actinic keratosis
ANTI-MIR	Antisense microRNA
ARVC	Arrhythmogenic right ventricular cardiomyopathy
ASPP	Apoptosis stimulating protein of p53
ATG	Autophagy-related gene gene
ATM	Ataxia telangiectasia mutated
BAX	BCL2-associated X protein
BCC	Basal cell carcinoma
BCL-2	B-cell lymphoma 2
BSA	Bovine Serum Albumin
C	Cytosine
Ca2+	Calcium
CDK	Cyclin dependent kinase
CDNA	Complementary DNA
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CI	Confidence interval
CMV	Cytomegalovirus
C-MYC	Avian myelocytomatosis viral oncogene homolog
CO2	Carbon dioxide
CPD	Cyclobutane pyrimidine dimers
cSCC	Cutaneous squamous cell carcinoma
DAB	3,3’-Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA binding domain
DDH2O	Double-distilled water
DGCR8	DiGeorge syndrome chromosomal region 8
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
E2F1	E2F Transcription Factor 1
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial–mesenchymal transition
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
G	Guanine
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GFP	Green fluorescent protein
GTP	Guanosine-5'-triphosphate
GUS	β-glucuronidase
H	Hour
HA	Hemagglutinin
HEK293	Human Embryonic Kidney 293

HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
HRP	Horseradish peroxidase
iASPP	Inhibitor of apoptosis-stimulating protein of p53
IKK	IκB kinase
IRF6	Interferon regulatory factor 6
KDA	Kilodalton
l	Litre
LB	Luria Bertani
LC3	Microtubule-associated protein 1A/1B-light chain 3
LNA	Locked Nucleic Acid
M	Molar
MDM2	Mouse double minute 2 homolog
MET	Mesenchymal–epithelial transition
Min	Minute
MiRNA	MicroRNA
ml	Millilitres
mM	Millimolar
MOI	Multiplicity of infection
mRNA	Messenger RNA
MSI	Microsatellite instability
Na_2HPO_4	Disodium phosphate
$Na_3C_6H_5O_7$	Trisodium citrate
NaCl	Sodium chloride
NAOH	Sodium hydroxide
NF-KB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NHK	Normal human keratinocytes
NS	Not significant
NSCLC	Non-Small Cell Lung Cancer
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
PFA	Paraformaldehyde
PIG3	p53-inducible gene 3
Plk1	Polo-Like Kinase 1
PRE-MIR	Precursor miRNA
PUMA	p53 upregulated modulator of apoptosis
QRT-PCR	Quantitative real-time PCR
RaDAR	RanGDP/Ankyrin Repeat
RAI	RelA-associated inhibitor
RBP-J	Recombining binding protein suppressor of hairless
RISC	RNA-induced silencing complex
RITA	Reactivation of p53 and induction of tumor cell apoptosis
RNA	Ribonucleic Acid
RPM	Revolutions per minute
RT	Room Temperature
S	Second
SCC	Squamous cell carcinoma

SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SH3	SRC Homology 3 Domain
SHRNA	Small hairpin RNA
SIRNA	Small interfering ribonucleic acid
SNP	small nucleotide polymorphism
SPF	Sun Protection Factor
SSC	Saline sodium citrate
T	Thymine
TA	transactivation domain
TRNA	Transfer ribonucleic acid
UTR	Untranslated
UV	Ultraviolet
WT	Wild type
ZEB	Zinc Finger E-Box Binding Homeobox
ΔΔCt	Delta delta Ct
µ	Micron
µg	Microgram
µl	Microlitre
µM	Micromolar

Chapter 1: Introduction

1.1. The human skin

The human skin is a large multi-functional organ that plays a key role in protecting the body from a variety of potentially damaging environmental elements. The defensive barrier the skin forms acts as a safeguard from harmful pathogens, UV radiation and physical injury. In addition to this environmental protection, the skin is also capable of controlling fluid loss from the body and can regulate the temperature of the body due to its expansive blood supply and ability to excrete sweat (Fuchs, 2007).

The human epidermis is a stratified squamous epithelium which forms the outermost layer of the skin (Kalinin et al., 2002). Within the epidermis there are four main compartments: the basal layer, spinous layer, granular layer and stratum corneum (Figure 1.1) (Fuchs, 1990; Proksch et al., 2008). The basal layer of the skin sits on the basement membrane consisting of extracellular matrix (ECM) proteins and growth factors. The basement membrane anchors the epidermis to the dermis located below and can act as a barrier preventing tumour cells from metastasising through to the dermis and beyond (Liotta et al., 1980). The basal layer comprises rapidly dividing keratinocyte cells that differentiate as they migrate towards the spinous and granular layer of the skin. Here, keratinocytes produce both keratins 14 and 5 enabling the formation of the cytoskeleton (Byrne et al., 1994). Stem cells move from the basal layer towards the spinous and granular layers where they begin the process of terminal differentiation. During this stage there is a shift in keratin expression to keratin 1 and 10, these keratins form larger bundles of keratin filaments offering a more solid structure (Byrne et al., 1994). Once cells reach the stratum corneum they are committed to terminal differentiation and become flattened forming an impermeable barrier before being shed and replaced by cells below (Fuchs & Raghavan, 2002).

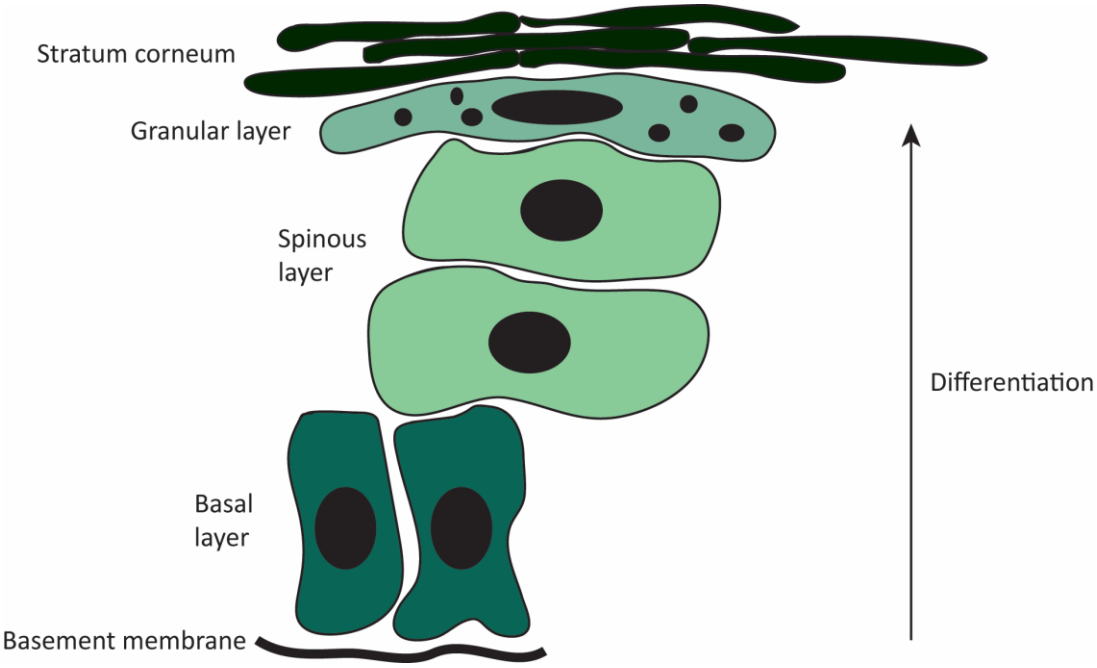


Figure 1.1. Epidermal layers of the skin. The human epidermis consists of four main sections: the basal layer, spinous layer, granular layer and the stratum corneum. The cells become increasingly differentiated as they migrate towards the surface of the skin. Figure adapted from Proksch et al. (2008).

1.2. Skin cancer

The three most common forms of human skin cancer are cutaneous melanoma, squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) (Rigel et al., 2005). Non-melanoma skin cancer, that includes SCC and BCC, is increasing and is estimated to be the cause of at least 100,000 new cases per year in the UK (Madan et al., 2010). Although more common, BCC is less likely to metastasise compared to SCC (Madan et al., 2010). Currently melanoma is responsible for 75% of skin cancer deaths in the Western world (Schadendorf and Hauschild, 2014). While non-melanoma skin cancer, as a whole, has a lower metastatic potential than melanoma, the large number of people affected by non-melanoma skin cancer mean there is not only a significant burden on patients but also on the healthcare services tasked with treating them (Eedy, 2000).

1.2.1. Causes of cutaneous SCC

SCC can develop on the head and neck, thyroid, oesophagus, lung, penis, prostate, vagina, cervix and bladder. However the most common area for SCC to develop is on sun exposed sites of the skin – cutaneous SCC (cSCC). The biggest risk factor for cSCC is UV light from the sun. In accordance with this, cSCC is found to develop at a higher incidence in fair skinned people and on sun exposed surfaces, for example, on the face and the back of hands (Figure 1.2) (Diepgen & Mahler, 2002; Healy et al., 2004). Proof of this can be found in the discovery that regular use of sun cream containing a high Sun Protection Factor (SPF) can result in a decline of cSCC cases and that tanning devices can cause a 2.5-fold increase in cSCC (Thompson, 1993; Samarasinghe & Madan, 2012). Darker skinned people are also at risk of cSCC but to a much lesser extent, however, this is heightened in areas of the skin with preexisting inflammatory skin conditions, burn injuries or trauma e.g., scars (Diepgen & Mahler, 2002). Additionally, a new observation worth noting is the increasing incidence of cSCC in the younger population in traditional 'non-sun exposed' areas, for example the trunk. Evidence suggests a potential socioeconomic role for this increase, that is, an increased affluence potentially allows for more leisure-related sun exposure (Deady et al., 2014).

Figure 1.2. Clinical presentation of cSCC. Images of patients diagnosed with cSCC. cSCC predominantly develops on sun exposed surfaces of the face, ear and back of hand. Clinical photos kindly provided by Prof Catherine Harwood.

At present, UVB, with a wavelength of 290-320 nm, is considered the prime source of UV radiation to induce skin cancer (Pfeifer et al., 2005). UVB causes direct DNA damage and can create cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts [(6-4)PPs] causing CC→TT and C→T transitions (Yoon et al., 2000). If not repaired correctly prior to DNA replication, these transition mutations can be incorporated into the DNA sequence. In the case of patients with xeroderma pigmentosum or patients suffering from other disorders containing compromised DNA repair systems, these CPDs and (6-4)PPs are unable to be excised by DNA repair systems thus making them extremely susceptible to skin cancer (Pfeifer et al., 2005). Although most of the research to date has focused on UVB as the main contributor to cSCC, more efforts are now focusing on the role

of UVA in cSCC. UVA has a longer wavelength (320–400 nm) than UVB and can cause indirect oxidative damage to DNA, via reactive oxygen species. The reactive oxygen species react with guanine to form 8-hydroxy-deoxyguanosine and in turn result in a mutation causing a G→T transversion (Bachelor & Bowden, 2004). Recently, a study has been performed linking cSCC developed in immunosuppressed patients to immunosuppressive drug ciclosporin A and UVA as both are able to induce the expression of activating transcription factor 3 which can downregulate p53 (Dziunycz et al., 2014). Prior to this study O'Donovan et al. (2005) suggested that immunosuppression of patients with azathioprine, a drug able to induce chronic oxidative stress, may actually be involved in inducing cancer. The relevance of this is described in section 1.2.3.

UV light however, is not the sole cause of cSCC and people who are immunosuppressed, for example, patients who have undergone organ transplantation, tend to be diagnosed with the disease approximately 15 years younger and display a more aggressive form of the disease than immunocompetent individuals (Harwood et al., 2013; Rosen et al., 2009). The risk of developing cSCC after organ transplantation is 65-250 fold higher than in non-transplant individuals (Madan et al., 2010). Taken together, these findings highlight the importance of the immune system in preventing the development of cSCC. In addition, patients undergoing cancer treatment for a different cancer to cSCC can often present cSCC due to the disruption of signalling pathways by cancer therapies, for example, patients undergoing treatment with BRAF inhibitors for metastatic melanoma have an increased chance of developing cSCC (Boussemart et al., 2013). Furthermore, individuals who have been exposed to chemical carcinogens (such as arsenic and tobacco amongst others) and those who suffer from genetic disorders, for example, xeroderma pigmentosum or albinism are all at high risk of developing cSCC (Diepgen & Mahler, 2002).

Not all cSCCs arise on sun exposed sites. Recently a lot of work has been performed attempting to prove a link between HPV and cSCC. The human papillomavirus (HPV) family contain several high-risk HPVs known to cause cancer. Nearly all cervical cancer is caused by HPV with 70% being caused by high-risk HPV-16 and 18 (Winer et al., 2006). Aside from cervical cancers several other cancers are associated with HPV including anal, oropharyngeal, vulvar, vaginal and penile cancers (Gillison et al., 2008) and also a proportion of head and neck SCC. PCR-based epidemiological studies have provided evidence for this link finding HPV DNA in over 80% of immunosuppressed and 30% of immunocompetent cSCCs (Harwood and

Proby, 2002). Although this, along with several other studies, has shown a high incidence of human papillomavirus infection in cSCC, given that a high proportion of 'normal' skin (ranging from 42-87%) also harbours the infection, the role for HPV in cSCC remains unclear (Aldabagh et al., 2013).

1.2.2. Progression of cSCC

cSCC occurs in the epidermis of the skin and arises due to the overproliferation and increased invasiveness of keratinocytes (Ratushny et al., 2012). cSCC tumours are formed by an initial thickening of the skin leading to an indurated plaque. These can present as papules, nodules or ulcerated lesions (Vivier, 2002). cSCC cases can arise from a premalignant lesion known as actinic keratosis (AK), however not all AKs progress to cSCC (Criscione et al., 2009). Typically, it has been suggested that patients with less than five AK lesions have a less than 1% chance of progressing to cSCC. In contrast, patients with 20 or more AK lesions have a 20% chance of progressing to cSCC (Ratushny et al., 2012). Prior to invasive cSCC, preinvasive stage of cSCC termed carcinoma-in-situ or Bowen's disease may form (Figure 1.3).

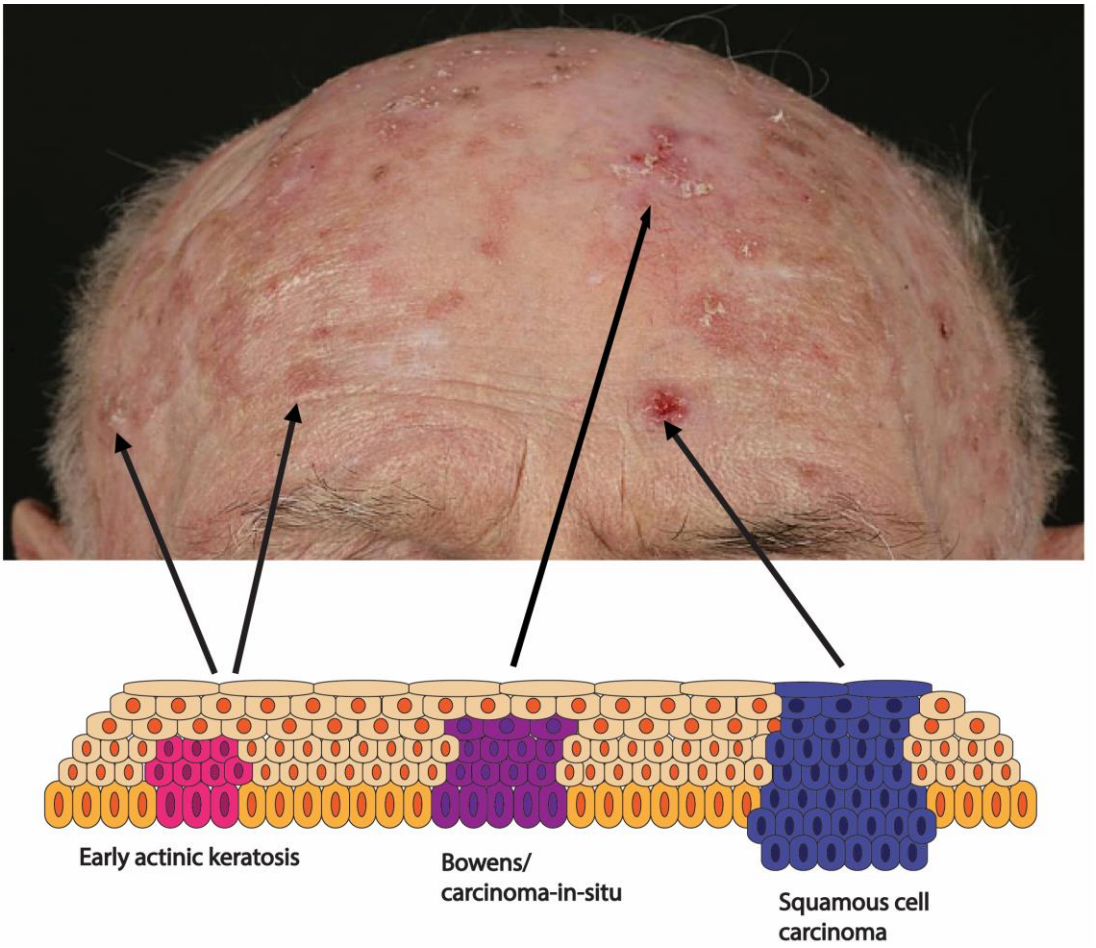


Figure 1.3. Progression of cSCC. Patient diagnosed with three different stages of cSCC. Clinical photo kindly provided by Prof Catherine Harwood.

cSCC can be grouped into well, moderate and poorly differentiated based on the differentiation status of the cells in the tumour (Figure 1.4). Poorly differentiated tumours are usually more aggressive and have a higher risk of metastasising. Other high risk factors that can determine the incidence of metastasis include the immunosuppression status of the patients and the initial location of the tumour, for example tumours located on the ear or lip have a higher chance of metastasising (Brantsch et al., 2008). In addition the size and depth of tumour affect the aggressiveness - tumours ≤2 mm in depth rarely metastasise, those >6 mm have a metastatic rate of 16% (Breuninger et al., 1990; Brantsch et al., 2008). The most common cause of cSCC related deaths is due to the tumour metastasizing.

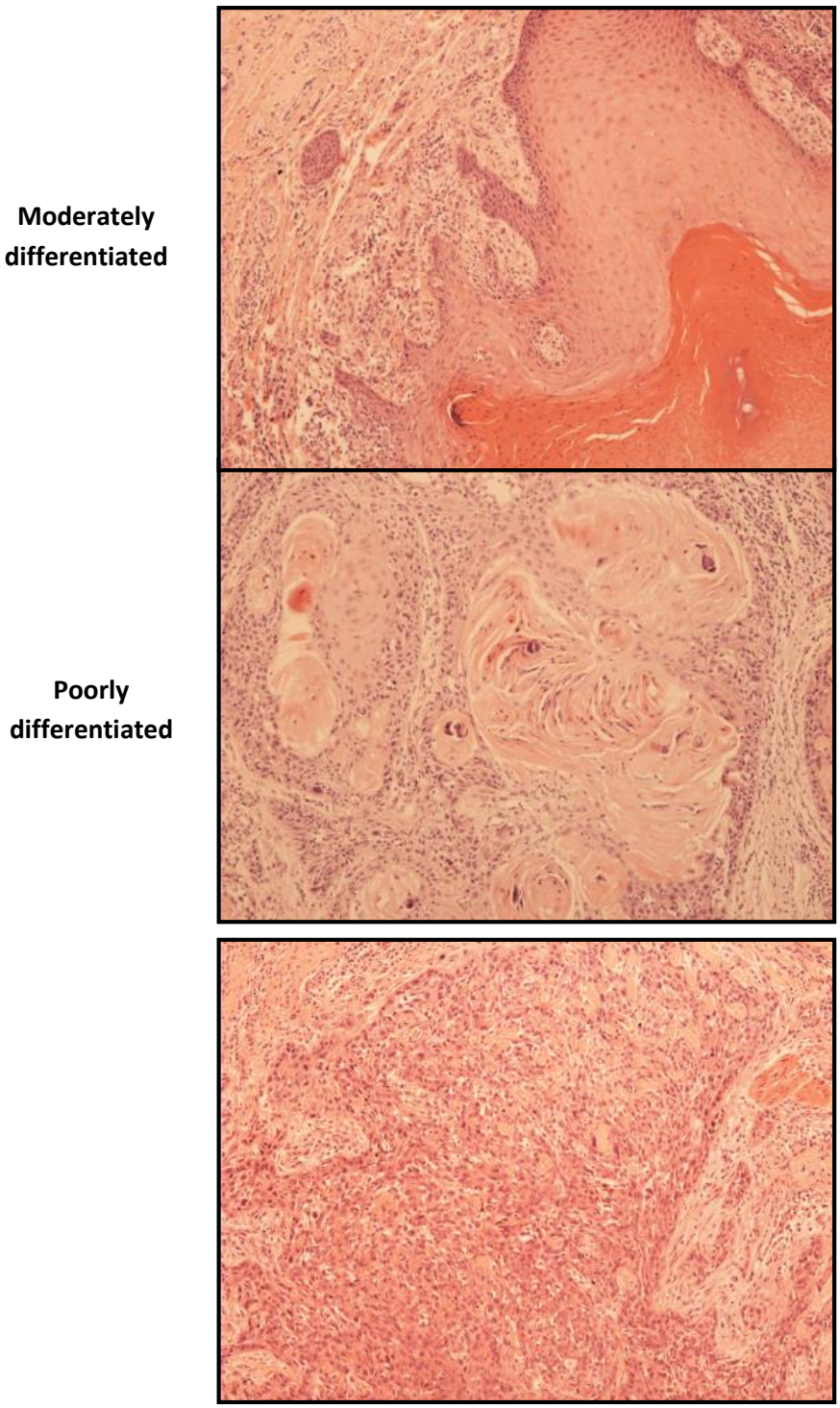


Figure 1.4. Haematoxylin & eosin sections demonstrating the differentiation statuses of cSCC. In well differentiated tumours, tumour cells are abnormal but tend to resemble normal keratinocytes. Well differentiated tumours are similar to the normal epidermis having basal cells located at the border of the tumour with cells maturing/differentiating towards the central tumour mass. The centre of a well differentiated tumour mass contains 'keratinous pearls'. Poorly differentiated tumours on the other hand contain highly abnormal cells which do not fully differentiate and are able to invade deeper.

1.2.3. Genetic development of cSCC

Mutations in p53 are deemed to be an early event in cSCC formation (Boukamp, 2005). Transition mutations can be found in a high proportion of cSCC cases in the tumour suppressor gene, p53 (Brash et al., 1991). p53 is a crucial regulator of the cell cycle and apoptosis and it is hypothesised that this early occurrence of mutated p53 allows the cell to become more resistant to apoptosis in turn, allowing the cell to harbour additional genetic changes. Upon cell damage, p53 can either mediate DNA repair or activate apoptosis. When mutated, p53 loses its ability to protect the cell in this manner resulting in uncontrolled cell growth (Prives & Hall, 1999). Mutations in p53 have been widely observed in cSCC with 69% to over 90% of invasive cSCC containing this mutation (Ziegler et al., 1994; Samarasinghe & Madan, 2012). In addition to p53, another member of the p53 family, p63, has also been implicated in cSCC, though this is discussed later in the chapter.

cSCC is a genetically heterogeneous disease, and in addition to p53 mutations, many other mutations are required before a premalignant lesion becomes metastatic. Inactivation of *CDKN2A* gene, which encodes for tumour suppressor genes $p16^{INK4a}$ and $p14^{ARF}$ via different reading frames, was found to be prevalent in cSCC. Ordinarily, $p16^{INK4a}$ acts as a tumour suppressor inhibiting cyclin-dependent kinases from phosphorylating, and inactivating retinoblastoma protein. $p14^{ARF}$ binds to MDM2 preventing degradation of p53. Both lead to prevention of cell cycle progression (Saridaki et al., 2003; Brown et al., 2004). Accordingly, inactivated *CDKN2A* leads to uncontrolled cell growth (Figure 1.5).

A further tumour suppressor gene which has been the attention of much recent study in cSCC is *Notch 1*, with approximately 60% of cSCC harbouring a Notch 1 mutation (Forbes et al., 2010). The Notch family consists of four receptors: Notch 1, 2, 3 and 4. The Notch receptors are transmembrane receptors that upon ligand binding to the outer section of the receptor become cleaved and the intracellular (and now active) domain is released into the cell and travels to the nucleus. Once in the nucleus, activated Notch binds to DNA-binding protein RBP-J and is able to activate the transcription of a variety of different genes (South et al., 2012). In the skin, Notch can activate genes involved in exiting the cell cycle and the promotion of differentiation and thus functions as a tumour suppressor (Nguyen et al., 2006). Nguyen et al., (2006) showed that Notch1 is able to suppress p63 levels in keratinocytes and in turn high levels of p63 are able to inhibit the activity of Notch1 (Nguyen et al., 2006). In addition, IRF6, a target of Notch1, together with Notch1, has tumour

suppressive functions and is able to inhibit p63 (Restivo et al., 2011). In cSCC decreased IRF6 and Notch1 signalling can result in the upregulation of p63 expression (Restivo et al., 2011). Thus it is unsurprising that in cSCC both Notch1 and IRF6 are mutated (Figure 1.5).

Mouse models depicting cSCC can have a high frequency of activating mutations in the oncogene, *RAS*. In contrast, studies in human cSCC models found that RAS mutations were present in human cSCC but at a lower incidence of around 10-20% (Boukamp, 2005). Further oncogenes implicated in the molecular pathogenesis of SCC include amplification of c-myc and activation of EGFR, a negative regulator of Notch 1 (Pelisson et al., 1996; Kolev et al., 2008).

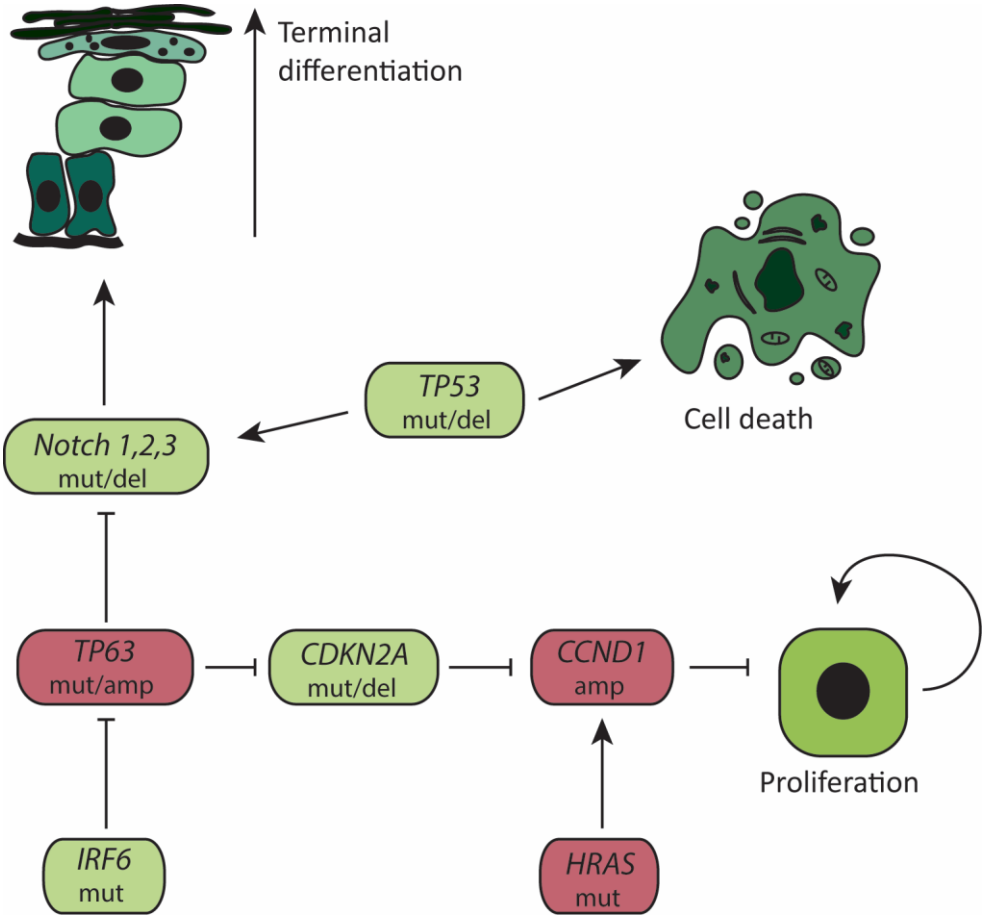


Figure 1.5. Genetics of SCC. Proposed diagram of the molecular pathways of cSCC. Green denotes a loss of function; red denotes a gain of function. Genes harbouring mutations (mut), amplifications (amp), or deletions (del) are shown. Figure adapted from Stransky et al. (2011).

1.2.4. Chromosome abnormalities in cSCC

Due to the genetic heterogeneous profile of cSCC the key events in cSCC tumourogenesis are not clearly defined. In order to refine this several studies have published data exploring recurrent aberrations on chromosomes of cSCC samples (Quinn et al., 1994a; Quinn et al., 1994b; Popp et al., 2000; Ashton et al., 2003; Clausen et al., 2006; Purdie et al., 2007; Purdie et al., 2009). Currently several chromosomal aberrations have also been implicated in cSCC including loss of heterozygosity at positions 9p, 3p, 2q, 8p, 13 and 17p and gain at positions 3q and 8q (Quinn et al., 1994a; Quinn et al., 1994b; Popp et al., 2000; Ashton et al., 2003; Clausen et al., 2006; Purdie et al., 2007; Purdie et al., 2009). These studies were carried out using a variety of different techniques such as comparative genomic hybridisation, microsatellite instability (MSI) mapping and more recently higher resolution single nucleotide polymorphism (SNP) microarrays showing a high consistency between the techniques. Purdie and colleagues (2009) investigated these chromosomal aberrations further finding a correlation with the differentiation status of the tumour. Well-differentiated tumours had fewer chromosomal aberrations compared to moderately and poorly differentiated tumours.

Recent data has also implicated the importance of telomere profile in cSCC. Leufke et al. (2014) discovered a relationship between short/homogeneous telomeres and a simple karyotype profile versus a more complex karyotype with long/heterogeneous telomeres. These two subtypes of telomere length suggest two different tumour initiation mechanisms.

1.2.5. MicroRNA development of cSCC

MicroRNA are small non-coding sections of RNA, usually 21-24 nucleotides, which are able to repress the translation or initiate the degradation of their target gene. MicroRNA play a crucial role in mammalian regulation of genes and are involved in the vast majority of human cellular processes (Filipowicz et al., 2008). Each microRNA has the capacity to target multiple genes. MicroRNA is synthesised by the process summarised in Figure 1.6. RNA polymerase II facilitates the transcription of a primary miRNA transcript. From here the Drosha/DiGeorge syndrome chromosomal region 8 (DGCR8) microprocessor complex cleaves the primary miRNA transcript into a precursor miRNA transcript via the formation of a hairpin loop. The precursor miRNA transcript is then exported from the nucleus to the cytoplasm by GTP-mediated exportin-5. Once in the cytoplasm the dicer complex attaches to the pre-miRNA

and undertakes additional cleavage of the pre-miRNA to form a double-stranded section of RNA containing a mature miRNA strand and a passenger miRNA strand. The mature miRNA strand becomes integrated into the RNA-induced silencing complex (RISC) whilst the passenger miRNA strand is degraded. This complex can now bind to its complementary mRNA resulting in the inhibition of translation of the target gene or its degradation (Filipowicz et al., 2008; Davis-Dusenbery & Hata, 2010).

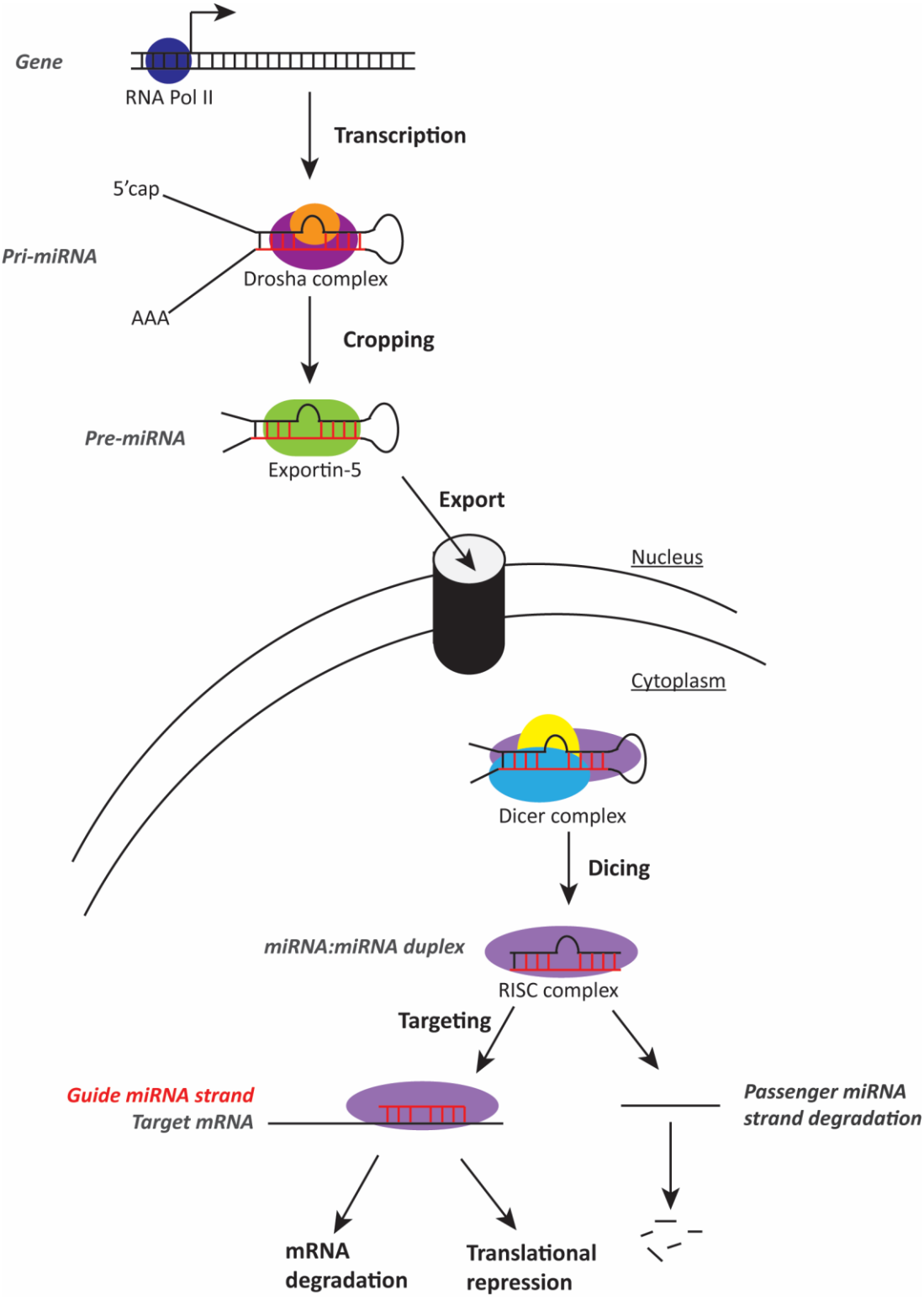


Figure 1.6. MicroRNA synthesis. Primary miRNA transcripts are transcribed in the nucleus by RNA polymerase II before being processed by drosha and exported to the cytoplasm via Exportin-5. In the cytoplasm the pre-miRNA is processed by dicer then subsequently integrated into the RISC complex where it is processed further to produce a mature miRNA. Figure adapted from Davis-Dusenbery & Hata (2010).

Research into microRNA in cancer, including cSCC, has increased in the last few years and several studies report the up- and down-regulation of microRNA in different disease types. Due to the ability of microRNA to bind to their complementary mRNA, microRNA can either be oncogenic or tumour suppressive depending on the gene they are repressing. For example, Table 1.1 shows this difference in regulation in recent studies that have been performed in cSCC. This is not an exhaustive list.

Table 1.1. MicroRNA implicated in cSCC.

Upregulation	Downregulation	Study
hsa-miR-21 hsa-miR-184	hsa-miR-203	Dziunycz et al., 2010
hsa-miR-135b hsa-miR-424 hsa-miR-766	hsa-miR-378 hsa-miR-145 hsa-miR-140-3p hsa-miR-30a hsa-miR-26a	Sand et al., 2012
-	hsa-miR-124 hsa-miR-214	Yamane et al., 2012
-	hsa-miR-125b	Xu et al., 2012 Zhou et al., 2013
hsa-miR-365	-	Zhou et al., 2013 Zhou et al., 2015
hsa-miR-21 hsa-miR-31 hsa-miR-205	-	Bruegger et al., 2013
-	hsa-miR-193b hsa-miR-365a	Gastaldi et al., 2014
-	hsa-miR-199a-5p	Wang et al., 2014b Kim et al., 2015a
hsa-miR-31	-	Wang et al., 2014a
-	hsa-miR-20a	Zhou et al., 2014
hsa-miR-135b	-	Olasz et al., 2015

MicroRNA expression in cSCC has been further researched comparing differences in the levels of microRNA between primary cSCC and metastatic cSCC. A recent publication found up-regulation of *miR-4286, miR-200a-3p* and *miR-148-3p in metastatic cSCC compared to primary cSCC* and down-regulation of *miR-1915-3p, miR-205-5p, miR-4516* and *miR-150-5p*

*(*Gillespie et al., 2015). These recent findings may provide useful biomarkers for metastatic cSCC.

1.2.6. Management of cSCC

Non-invasive treatments for non-metastatic cSCC are scarce and there are limited treatment options for metastatic SCC (Madan et al., 2010). At present, surgery is the most common form of treatment for non-metastatic cSCC. Unsurprisingly, however, this can come with disadvantages including scarring, a need for skin grafts and a poor cosmetic outcome. Standard surgery, that is surgery followed by a post-operative pathologic assessment, has a high success rate however excision of difficult areas may require the need for plastic reconstruction (Stratigos et al., 2015). Alternatively a more precise technique named Mohs micrographic surgery allowing microscopic examination of the excision margins during the surgery, removing one layer of tissue at a time allows the surgeon to ensure complete removal of the tumour whilst conserving normal tissue (Stratigos et al., 2015).

Radiotherapy may be used as an alternative to surgery in cases where surgery is not an option i.e., if the patient has inoperable cSCC or refuses surgery, or may be used as an adjuvant therapy. Treatments such as cryotherapy or electrodesiccation and curettage may be used but to a much lesser extent due to their lack of specificity. Photodynamic therapy with aminolevulinic acid or methyl aminolevulinate is another alternative treatment that uses a photosensitising agent which can be taken up by cancer cells. Upon light exposure the photosensitive agent reacts with oxygen present in the cell, killing the cell. Although specific and less invasive than surgery, photodynamic therapy is unable to target invasive tumours but is used for Bowen's disease and AK (Stratigos et al., 2015).

Chemotherapeutic drugs including cisplatin, doxorubicin, 5-flurouracil and bleomycin, have all been trialled for the treatment of advanced cSCC (Guthrie et al., 1990; Sadek et al., 1990; Khansur et al., 1991; Cartei et al., 2000). These drugs have been used both alone and in combination but with limited clinical activity (Cranmer et al., 2010). Potentially promising results have however been shown using a more targeted approach with EGFR inhibitors, particularly in metastatic cSCC where overexpression of EGFR can be observed (Maubec et al., 2005; Maubec et al., 2011; Lewis et al., 2012).

1.3. iASPP

1.3.1. Background

iASPP is part of the 'apoptosis stimulating proteins of p53' (ASPP) family that consists of three members: ASPP1, ASPP2 and iASPP. All of these members are characterised by their ability to interact with the tumour suppressor, p53 (Trigiante & Lu, 2006). Whilst ASPP1 and ASPP2 play a pro-apoptotic role with p53, enhancing its binding to pro-apoptotic proteins (Samuels-Lev et al., 2001), iASPP, the inhibitory member of the ASPP family, plays an oncogenic role by inhibiting p53-induced apoptosis (Bergamaschi et al., 2003 and 2006). IASPP was first discovered in 1999 by Yang et al., as a p65 rel A binding protein (Rel A- Associated- Inhibitor, RAI) and was then subsequently identified in *Caenorhabditis elegans* and in humans as iASPP, the most evolutionary conserved inhibitor of p53, encoded by the *PPP1R13L* gene (Bergamaschi et al., 2003).

iASPP is largely expressed in epithelial cells and therefore can be found in the skin and heart. iASPP expression has also recently been observed in the retinal ganglion cells in the central nervous system (Wilson et al., 2014). Mouse models containing a deletion mutation in *PPP1R13L* present an altered phenotype to wild-type mice with cardiac and skin abnormalities that lead to the development of a rapidly progressive cardiomyopathy, open eyelids at birth and wavy hair (Herron et al*.,* 2005; Toonen et al., 2012). Cattle that contain a frameshift mutation in *PPP1R13L* also exhibit similar phenotypes to the deletion mice including cardiomyopathy and a woolly coat (Simpson et al*.*, 2009).

1.3.2. Structure & location

iASPP was initially discovered as a 477 amino acid protein but the full length of iASPP was later established as an 828 amino acid protein (Yang et al., 1999; Slee et al., 2004). The ASPP family members all contain a similar C-terminal domain consisting of a proline-rich region, four ankyrin repeats and a SH3 domain (Figure 1.7). It is this C-terminal domain which proves to be important for the nuclear localisation of iASPP and enables iASPP to bind to its binding partners (Slee et al., 2004). p53 preferentially binds to this domain along with p65/Rel A and Bcl-2 among others (Gorina & Pavletich, 1996; Sullivan & Lu 2007). Later studies have also demonstrated the ability of iASPP to interact with the additional members of the p53 family, p63 and p73 (Robinson et al., 2008; Chikh et al., 2011; Notari et al., 2011). Although full length iASPP is largely located in the cytoplasm, many of the proteins iASPP is able to interact

with are predominantly located in the nucleus e.g., p53, p63 and NF-κB, which potentially indicates a vital role for iASPP cellular localisation signals (Trigiante & Lu, 2006). iASPP does not contain a nuclear localisation signal, however is still able to enter the nucleus (Slee et al., 2004). Recent work demonstrated that iASPP is able to enter the nucleus via an importin-independent mechanism - the RaDAR (RanGDP/Ankyrin repeat) pathway. The Ankyrin repeats present in the C-terminus of iASPP, specifically two adjacent 13th hydrophobic residues harbour a code enabling RanGDP to bind and complex with nuclear transport factor 2 resulting in the import of the complex into the nucleus (Lu et al., 2014).

Some researchers have speculated that the important role iASPP plays in interacting with nuclear based transcription factors demonstrates that it is the nuclear form of iASPP that is the most active (Lu et al., 2013). Recent publications from both melanoma and prostate cancer show a strong association between the expression of a phosphorylated form of nuclear iASPP and metastatic disease (Lu et al., 2013; Morris et al., 2014). Taking the above information into account however, it is therefore difficult to explain why iASPP is highly expressed in the cytoplasm in a broad range of tumour types (Jiang et al., 2011; Liu et al., 2012; Cao et al., 2013). iASPP is distinct from its family members as it lacks the α-helical domain on its N-terminal region that its family members contain (Figure 1.7). The N-terminus of iASPP is important for its cytoplasmic location within the cell (Slee et al., 2004). In the normal skin, iASPP has mainly nuclear basal expression but becomes largely cytoplasmic in the differentiated epithelial cells (Notari et al., 2011).

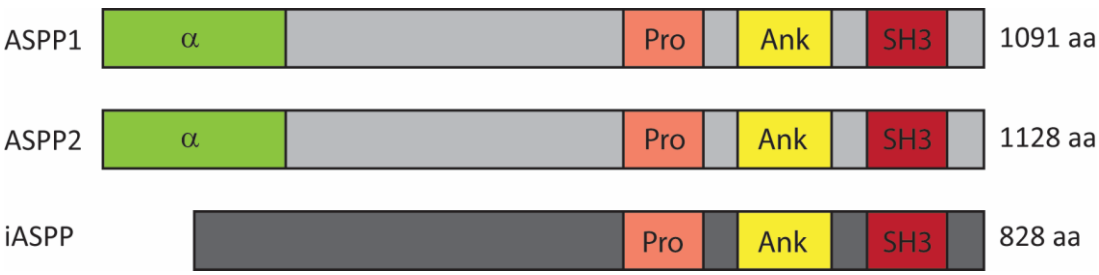


Figure 1.7. ASPP family structures. The three members of the ASPP family have similar structures. While ASPP1 and ASPP2 contain an α-helical domain, iASPP lacks this domain and contains fewer amino acids. Figure adapted from Sullivan & Lu (2007).

1.3.3. Function

1.3.3.1. Anti-apoptotic

Tumour suppressor gene *p53* is a crucial regulator of apoptosis (Vousden and Lu, 2002). Upon stimulation by ASPP family members, ASPP1 and ASPP2, p53 is able to bind to the promotors and transcriptionally activate proapoptotic genes (Samuels-Lev et al., 2001)**.** Despite the high sequence homology between the ASPP family, the third member of the ASPP family, iASPP, has an opposing function to ASPP1 and ASPP2 and is able to inhibit p53-induced apoptosis by binding to the p53 DNA binding domain via the c-terminal domain (Robinson et al., 2008). Currently, the anti-apoptotic activity of iASPP is well established in the field. Studies of iASPP in transformed cancer cells have demonstrated its role as a p53-dependent inhibitor of apoptosis with cells containing wild type p53 showing increased UV and cisplatin-induced apoptosis when treated with siRNA against iASPP (Bergamaschi et al., 2003). IASPP is able to inhibit p53-induced apoptosis by binding to p53 and preventing its transcriptional activity on proapoptotic BAX and PIG3 promoters (Figure 1.8) (Bergamaschi et al., 2003). A role for iASPP as a p53-independent inhibitor of apoptosis has also recently emerged. Using a p53-deficient cancer cell line, H1299, Cai et al. (2012b) were able to show iASPP binding to and inhibiting the transcriptional activity of p53 family members – p63 and p73 on proapoptotic genes.

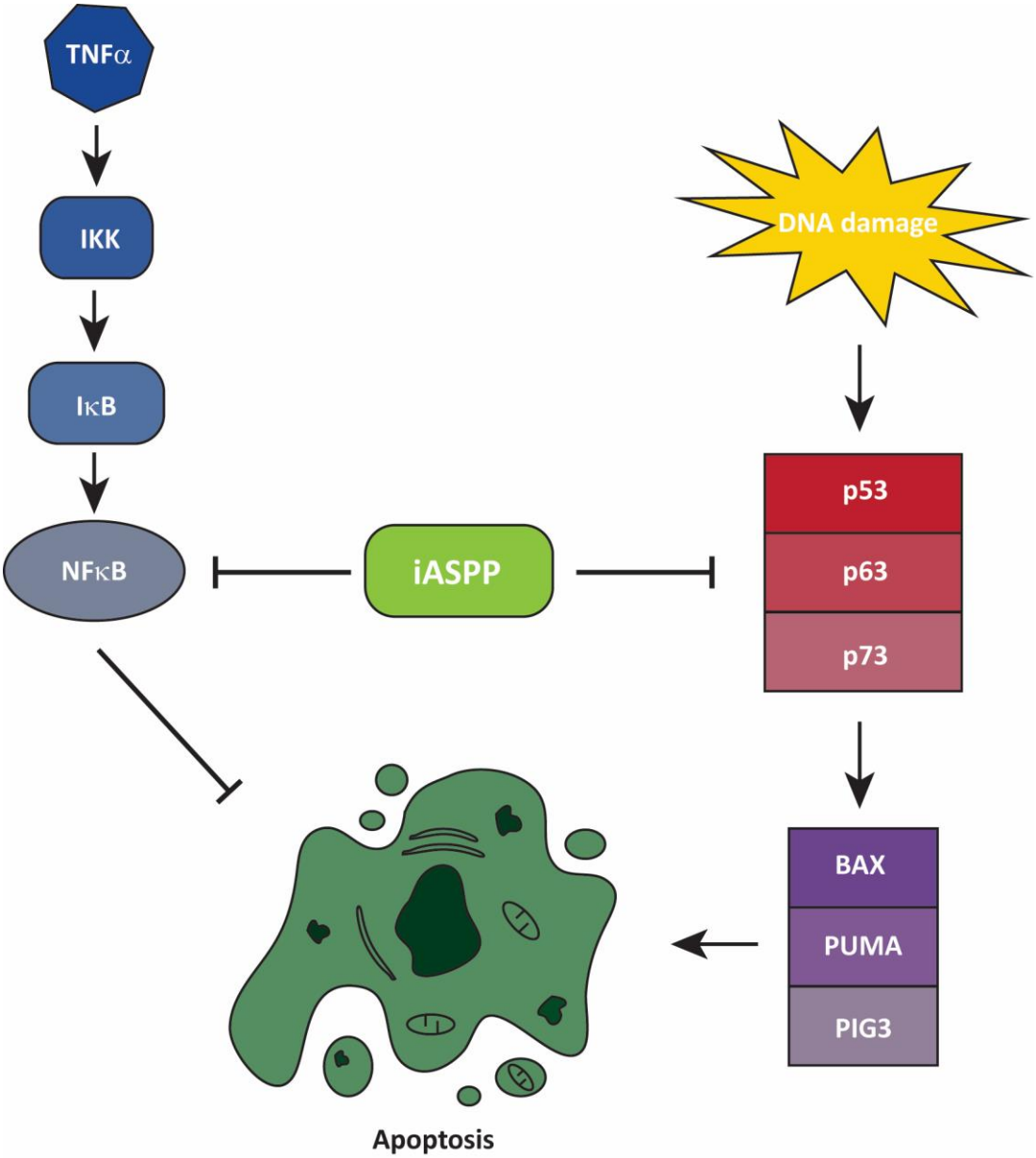


Figure 1.8. The contrasting roles of iASPP in the cell. Research has shown conflicting roles for iASPP's apoptotic activity in the cell. Largely, upon DNA damage in tumourigenic cell lines, iASPP is able to inhibit pro-apoptotic genes and thus is able to contribute to the anti-apoptotic behavior of the cell. In non-tumourigenic cell lines iASPP is able to inhibit anti-apoptotic NFκB activity leading to a pro-apoptotic outcome. Figure adapted from Trigiante & Lu (2006).

1.3.3.2. Pro-apoptotic

Before the oncogenic role of iASPP was characterised, iASPP was originally identified as a potential negative regulator of the NFκB signaling pathway, binding p65/rel A binding protein (Yang et al., 1999). P65/Rel A is a subunit of the NFκB complex which is involved in the immune and inflammatory responses of the cell and can inhibit apoptosis. Laska et al. (2007) demonstrated that in non-transformed cells, silenced for iASPP and treated with etoposide, there was a reduction of apoptosis and that treatment with an NFκB inhibitor on these cells reversed this effect. When repeated on transformed cells this effect did not occur suggesting that the pro-apoptotic effects of iASPP are limited to non-transformed cells (Figure 1.8). Conversely, data recently published using melanoma cell lines as a model also provides evidence for a pro-apoptotic role of iASPP, this time in transformed cell lines. In melanoma cell lines iASPP promotes apoptosis via the acetylation and thus stablisation of p53 and p73 isoform - TAp73, through acetyltransferases p300 and CBP (Kramer et al., 2015).

1.3.3.3. Inhibitor of autophagy

A recent study performed by our group (Chikh et al., 2014) also supports the notion presented by Laska et al. (2007) that iASPP does not always play an anti-apoptotic role in non-transformed cells. Chikh et al., 2014 have demonstrated another novel function of iASPP in HaCaT and N-TERT cells showing that silenced iASPP in keratinocytes did not cause an increase in apoptosis but rather triggered an increase in autophagy providing a role for iASPP as a novel autophagy inhibitor (Figure 1.9). HaCaT and N-TERT cells depleted of iASPP had decreased levels of Noxa, a pro-apoptotic member of the Bcl-2 family. When HaCaT and N-TERT cells were treated with UV to trigger apoptosis, cells depleted for iASPP did not show an increase in apoptosis as previously observed in several cancer models. Further investigation provided evidence that cells depleted of iASPP had increased expression of several markers of autophagy. Conversely, increased iASPP expression reduces the formation of the Atg5–Atg12–Atg16L1 complex preventing LC3 lipidation and therefore autophagososome formation in human keratinocytes (Mizushima et al., 2011).

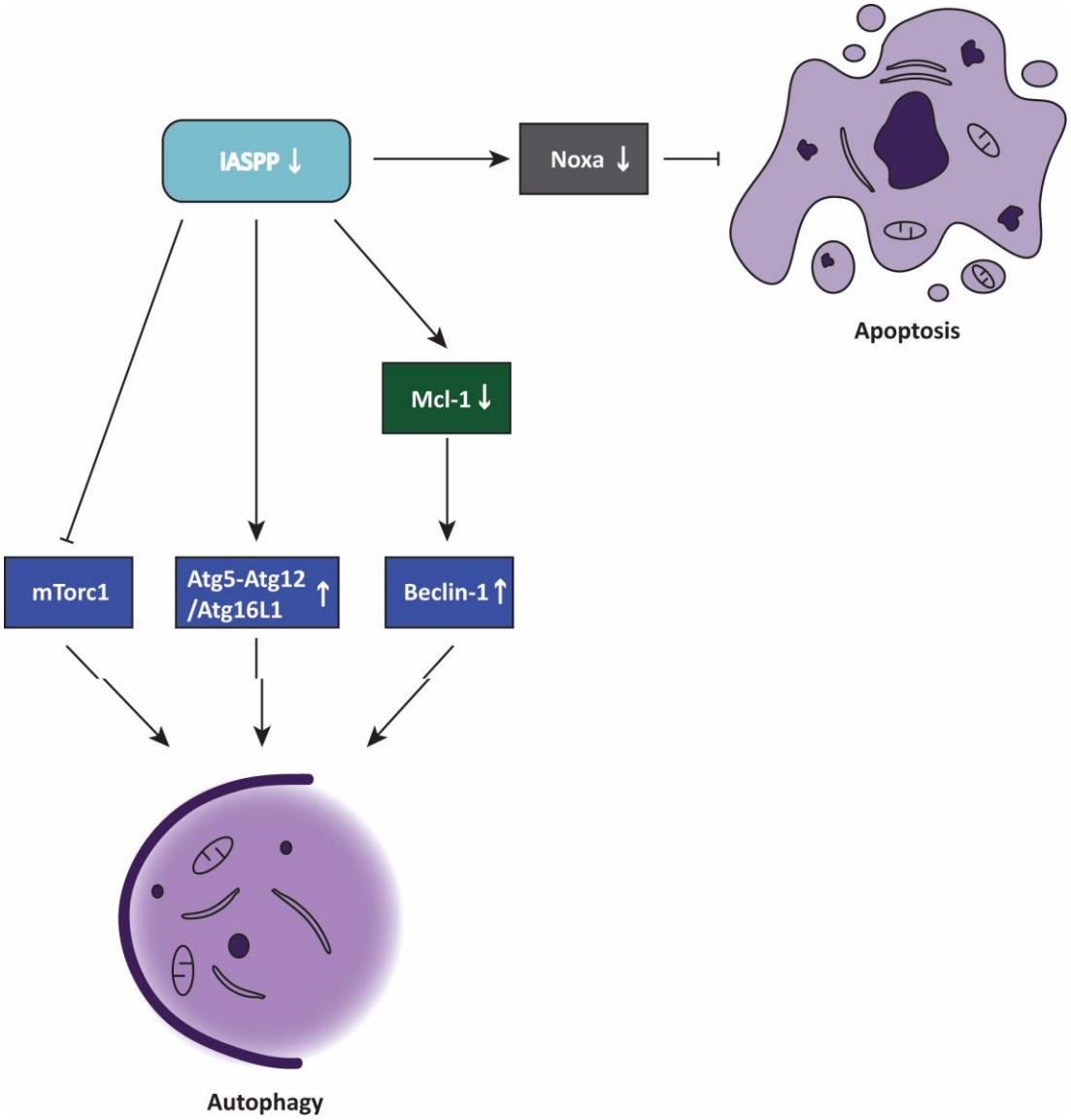


Figure 1.9. The role of iASPP in autophagy. In non-transformed cells silenced iASPP is able to decrease NOXA expression resulting in the inhibition of apoptosis. Furthermore, silenced iASPP deregulates mTORC signalling, a classical signalling pathway which normally controls the autophagy process and increases the interaction of ATG5/12 with ATG16L1 required for autophagosome formation leading to an increase in autophagy. Figure adapted from Chikh et al. (2014).

1.3.3.4. Cellular adhesion

The effects of iASPP on epidermal homeostasis have recently been investigated. iASPP is able to regulate epidermal adhesion in normal keratinocytes whereby depletion of iASPP from keratinocytes triggered a deregulation of the junctional complex integrity (Chikh et al., 2011). Upon silencing of iASPP, several genes involved in cell-matrix adhesion, for example β1 integrin, desmosmal and adhesion proteins including PERP and Claudin 1, amongst others were downregulated. The importance of iASPP in maintaining cell junctions is also clear in mouse cardiomyocytes where iASPP deficient mice develop arrhythmogenic right ventricular cardiomyopathy (ARVC) known to cause defects in desmosome integrity (Notari et al., 2015).

1.3.3.5. Proliferation/differentiation

iASPP is a promoter of proliferation and an inhibitor of differentiation. Cells depleted for iASPP display reduced proliferation caused by delayed cell-cycle progression. This decrease in proliferation was detected in HaCaT cells depleted for iASPP. Bromodeoxyuridine kinetic analysis showed that iASPP was able to alter cell-cycle progression, delaying entry into G1 phase. Complementing these data was the finding that cyclin D2 expression, necessary to permit the cells to cycle through G1/S phase, is reduced upon iASPP knockdown. iASPP regulation of proliferation is not limited to a non-tumour setting. Several papers support the notion of iASPP controlling cell proliferation in a cancer setting. Depletion of iASPP in prostate, bladder and gastric cancer cell lines, in addition to NSCLC, glioblastoma and hepatocellular carcinoma cells causes a negative effect on the cells' ability to proliferate (Pang et al., 2010; Lu et al., 2010; Zhang et al., 2011; Lin et al., 2011; Chen et al., 2011, Li et al., 2011; Lu et al., 2013; Morris et al., 2014; Wang et al., 2015a). In melanoma the HEDGEHOG/GLI-E2F1 axis positively modulates iASPP's ability to regulate cell proliferation (Pandolfi et al., 2015). Chen et al. (2014b) showed the negative effect iASPP had upon depletion from oral tongue squamous cell carcinoma cell lines. However, currently no reports have documented the effects of iASPP on cell proliferation in cSCC.

Although depletion of iASPP inhibits the proliferation of the cell, iASPP is also implicated in differentiation and stratification of the epidermis. When the normal epidermis is depleted of iASPP the thickness of the epidermis increases. This observation was correlated with an increase in the expression of both involucrin and loricrin, markers of keratinocyte terminal differentiation (Chikh et al., 2011). The latter effect has been further confirmed by a report using a transgenic mouse in which iASPP expression is controlled by the Cre/loxP

recombination system (Notari et al., 2011). In this mouse when iASPP was depleted the number of cells increased in the epidermis compared to control. As this study and previous studies have shown that iASPP deficiency reduces the proliferation of the cell, the observation of a thicker epidermis upon iASPP silencing was shown to occur due to an increase in differentiation (Notari et al., 2011).

1.3.3.6. Senescence

This reduction in proliferation observed in cells hinted at a possible role for iASPP in cellular senescence. Senescence was originally described as a process that limited the proliferation potential of cells (Hayflick, 1965). The p53 family members are able to control senescence via transactivating/repressing genes (Guo et al., 2009; Notari et al., 2011; Rufini et al., 2013). Using mouse embryonic fibroblast cells depleted for iASPP Notari et al. (2011) found these cells stained positive for β-galactosidase, a commonly used marker for senescence, confirming a role for iASPP in inhibiting cellular senescence.

1.3.4. iASPP regulation

At present there is a shortage of data concerning the upstream mechanisms of regulation of iASPP. The majority of research performed so far has instead focussed on the effects of iASPP in various transformed and non-transformed cell models. However, several studies have started to investigate the regulation of iASPP and are described below.

1.3.4.1. p63

Robinson et al. (2008) previously demonstrated that iASPP and p63 were able to bind based on structural evidence. Using solid-phase binding assays it was demonstrated that iASPP was also able to bind p63 at a 3-fold higher affinity than ASPP2 (Robinson et al., 2008). Based on the above information and the finding that iASPP and p63 are able to colocalise in the normal skin, a series of experiments by our group were performed in the stratified epithelia and linked iASPP to p63 and, in turn, demonstrated a feedback loop involving two microRNAs (Chikh et al. 2011). Chikh et al., (2011) proved, via chromatin immunoprecipitation using HaCaT cells, that p63 was a direct transcriptional regulator of iASPP, able to bind directly to the promoter of iASPP. HEK293 cells over expressing p63 showed a sequential increase of iASPP expression, confirming the ability of p63 to positively regulate iASPP, whilst silencing

of p63 in primary keratinocytes decreased the expression of iASPP at both the protein and mRNA level.

1.3.4.2. Phosphorylation

Recently it was demonstrated in human melanoma cells that iASPP could form homodimers via its N-terminus interacting with its C-terminus. These homodimers reside in this form in the cytoplasm. Upon phosphorylation at S84/S113 by cyclin B1/CDK1, iASPP is unable to form homodimers and can translocate to the nucleus and expose its p53 binding site, leading to a decrease in p53 activity (Lu et al., 2013). In accordance with this theory, melanoma cells exhibited a high phosphorylated nuclear iASPP expression profile. Prostate cancer also contains phosphorylated nuclear iASPP and this phosphorylated form of nuclear iASPP correlates with the metastatic disease (Morris et al., 2014).

1.3.4.3. Hedgehog signalling

The Hedgehog signalling pathway is frequently activated in melanoma and is able to impair p53 function (Stecca et al., 2007). Using the recent finding that melanoma contains phosphorylated iASPP able to inhibit wild-type p53, Pandolifi et al., (2015) investigated whether the Hedgehog signalling pathway was regulating iASPP. It was discovered that Hedgehog pathway directly regulates E2F1 which, in turn, positively regulates iASPP expression, enabling the inhibition of p53 signalling (Figure 1.10). Additionally, the hedgehog pathway is able to increase the levels of cyclin B1 and CDK1 providing further activation of iASPP via phosphorylation as described in the previous section (Pandolifi et al., 2015).

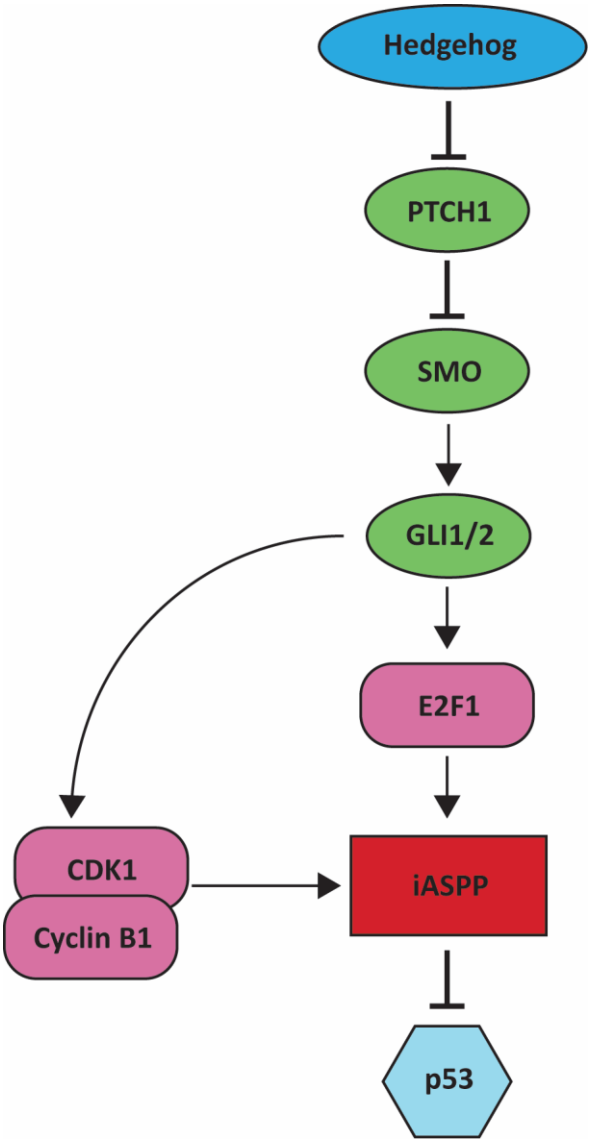


Figure 1.10. Regulation of iASPP in melanoma. E2F1, a downstream target of Hedgehog signaling, is able to regulate iASPP expression, in turn inactivating p53. Hedgehog signalling effectors GLI1 and GLI2 regulate cyclin B1/CDK1, responsible for the phosphorylation and activation of iASPP. Figure adapted from Pandolifi et al. (2015).

1.3.4.4. MicroRNA

Until recently there was a lack of information regarding the regulation of iASPP by microRNA. Several studies however, have now been performed indicating a role for microRNA in the regulation of iASPP. To date four publications have demonstrated the ability of miR-124 to downregulate iASPP in a variety of disease models (Zhao et al., 2013; Liu et al., 2013a; Lui et al., 2013b; Chen et al., 2014a). In prostate, glioblastoma and colorectal cancer cell lines luciferase assays confirmed the direct interaction of miR-124 with iASPP (Liu et al., 2013a; Zhao et al., 2013; Chen et al., 2014a). The result of this regulation of iASPP by miR-124 was a decrease in cell proliferation in all cell line models tested. Additionally, miR-124 was proven to directly target iASPP and promote neuronal death after cerebral ischemia in mouse brain samples (Lui et al., 2013). Interestingly in cSCC, the expression of miR-124 was significantly downregulated both in vitro and in vivo (Yamane et al., 2013).

1.3.4. iASPP and cancer

As described above, iASPP has been well-documented as an inhibitor of both p53-dependent and p53-independent apoptosis. Due to this oncogenic activity in the cell it is therefore to be expected that iASPP is involved in a cancer setting. As mentioned previously, Bergamaschi et al. (2003 & 2006) demonstrated that iASPP is upregulated in human breast carcinoma. Cell lines depleted for iASPP but containing wild type p53 showed an increase in apoptosis when treated with increasing UV and cisplatin concentrations. Similarly, Zhang et al. (2005) showed an upregulation of iASPP expression in acute leukaemia compared to normal bone marrow cells. Moreover, several studies have correlated the upregulation of iASPP expression to a cancer phenotype. The majority of these studies into iASPP and cancer have been observational, that is, detecting high levels of iASPP in cancer cell lines and clinical samples. Regardless, studies carried out in colorectal adenoma and carcinoma, leukaemia, hepatocellular carcinoma, prostate and ovarian cancers, non-small cell lung carcinoma, glioblastoma and endometrial endometrioid adenocarcinoma all provide evidence that iASPP is an oncogenic protein (Saebo et al., 2006; Liu et al., 2009; Lu et al., 2010; Chen et al., 2010; Jiang et al., 2011; Zhang et al., 2011; Li et al., 2011; Liu et al., 2010; Lin et al., 2012).

More recent publications into the role of iASPP in cancer have delved further. The role of iASPP in melanoma has been extensively studied by Lu et al., (2013). As discussed earlier, in melanoma, iASPP resides as a dimer in the cytoplasm until its phosphorylation by cyclin B1/CDK1 allowing monomeric entry into the nucleus. Once in the nucleus iASPP is able to

inactivate wild type p53. In agreement with these results is the finding that high nuclear iASPP expression is associated with poor patient survival and metastatic melanoma (Lu et al., 2013). Furthermore treatment of mice containing highly aggressive melanoma with JNJ-7706621, a CDK1 inhibitor, reduced both the tumour size and weight (Lu et al., 2013). Follow-up work was carried out in prostate cancer, where phosphorylated nuclear iASPP was detected in prostate cancer cell lines. In this cell model however, high cytoplasmic iASPP expression was also associated with metastasis and prostate-cancer specific death.

High iASPP expression has also recently been discovered in head and neck, and cervical squamous cell carcinomas (Liu et al., 2012; Cao et al., 2013). In head and neck SCC both protein and mRNA levels of iASPP were upregulated in HNSCC tissues and cell lines. In particular cytoplasmic iASPP was an independent prognostic factor for HNSCC patients whilst the expression of both nuclear and cytoplasmic iASPP correlated with shorter disease free survival (Liu et al., 2012). Conversely in cervical SCC, although cytoplasmic iASPP expression was also detected in the tumours, only nuclear iASPP expression correlated with shorter disease free survival and was an independent prognostic factor for overall survival (Cao et al., 2013).

1.3.5. iASPP and chemoresistance

The role of iASPP in chemoresistance was also researched in ovarian cancer where a strong link between iASPP and chemoresistance to paclitaxel was detected in ovarian cancer (Jiang et al., 2011). In ovarian cancer cell lines paclitaxel induces mitotic catastrophes. Through the discovery that iASPP was able to inhibit mitotic catastrophes in cells with mutated or loss of p53, Jiang et al. (2011) speculated that iASPP was able to inhibit mitotic catastrophes in a p53 independent manner. Additionally the data show that iASPP elevated levels of separase – a cysteine protease involved in the mitotic catastrophe - by degrading securin, an inhibitor of separase. Cao et al., (2013) also found a strong correlation between high iASPP expression and increased chemoresistance and radioresistance in cervical cancer. Interestingly, Cao et al. (2013) discovered a role for nuclear iASPP in chemoresistance whereas Jiang et al. (2011) found predominantly cytoplasmic expression of iASPP in the tumour and thus demonstrated a potential link between cytoplasmic iASPP expression and chemoresistance. Recent contradictory data come from melanoma cells demonstrating that in cells depleted for iASPP, breast metastasis suppressor 1 - an E3 ubiquitin ligase - is able to destablise

acetyltransferases p300 and CBP (responsible for the stablisation of p53 and TAp73) and actually promote chemoresistance (Kramer et al., 2015).

1.3.6. iASPP therapeutics

The predominant role of iASPP as an oncogene in cancer would suggest that it could be manipulated for therapeutic advantage. Another key aspect of iASPP that would make it an attractive candidate to target the tumour is the fact that iASPP is rarely mutated in cancer. Targeting a drug towards p53 for example, particularly in cSCC would be challenging due to its high mutation rate. Targeting iASPP may enable the reactivation of the apoptotic pathway that has been lost in so many cancers and thus prove to be a key target. On the other hand, targeting iASPP directly may not be the most ideal solution due to its importance for cell homeostasis, particularly in the skin. Therefore designing therapeutics aimed downstream of iASPP could provide better targets. Additionally, emerging evidence seems to suggest the effects of iASPP on the cell may be location and cell type specific. These are additional important factors that need taking into account.

At present a handful of drugs have been discovered to have an effect on iASPP. RITA (reactivation of p53 and induction of tumour cell apoptosis) can prevent p53 from binding to iASPP and thus increase apoptosis in tumour cells (Issaeva et al., 2004). Simvastatin, a cholesterol-lowering drug, decreases the expression of iASPP and increases the levels of apoptosis in melanoma cells (Wang et al., 2013). In melanoma cells it has been shown that nuclear iASPP is phosphorylated by Cyclin B1/CDK1. Treatment with JNJ-7706621, a CDK1 inhibitor, results in a better p53-dependent apoptotic response, particularly when used in combination with Nutlin-3 (an mdm2 inhibitor) (Lu et al., 2013). Furthermore a small peptide derived from p53 linker region, A34, has been developed and is able to bind to iASPP allowing the release of p53 to bind to the promoters of pro-apoptotic genes in U2OS osteosarcoma and MKN-45 gastric cancer cells (Qui et al., 2015). Injection of MKN-45 cells into nude mice followed by treatment A34 slowed tumour growth. MiR-124 recently found to regulate iASPP is also a promising therapeutic target (Zhao et al., 2013; Liu et al., 2013a; Lui et al., 2013; Chen et al., 2014a).

1.4 p63

1.4.1. Background

p63 is part of the p53 family and was first discovered by Yang et al. (1998) to be expressed in the basal epithelial cells in the epidermis of the breast, skin, cervix, urothelium and prostate, amongst others. Initial studies into p63 generated a lot of confusion due to seemingly conflicting roles of p63 in various cellular processes, including cancer. It has since been discovered that p63 is able to play conflicting roles in a variety of cancers due to its many different isoforms (Su et al., 2013).

1.4.2. Structure & location

p63 is encoded by the *Tp63* gene which is located on chromosome 3q27-28. Like all the members of the p53 family, the p63 isoforms have the same core structures in common: a transactivation domain, a DNA binding domain and oligomerization domain, and are able to form tetramers via their oligomerization domains to enable their stability (Figure 1.11) (Yang et al., 1998).

p63 is found in many different isoforms as a result of alternative splicing and alternative promoters. Two different promoters give rise to two main subgroups of p63: ΔNp63 and TAp63. The TAp63 subgroup contains a transactivation domain, a DNA binding domain, and oligomerization domain. The ΔNp63 subgroup lacks the transactivation domain present in the other members of the family (Murray-Zmijewski et al., 2006).

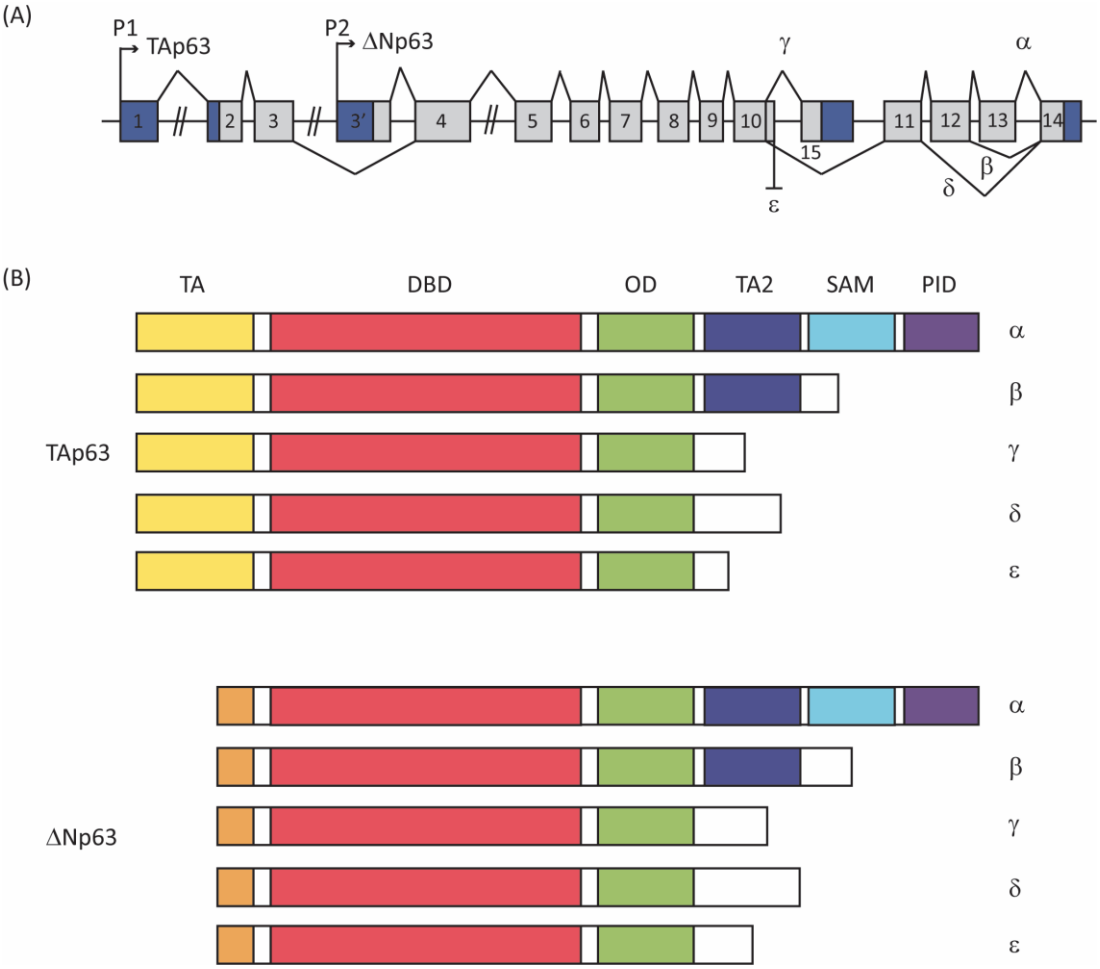


Figure 1.11. Structure of p63 isoforms. (A) The human p63 gene structure encoded by 16 exons with alternative splicing promoter sites (P1 and P2) giving rise to TA and ΔN isoforms and alternative splicing sites giving rise to splice variants. (B) The two subgroups of p63; TAp63 and ΔNp63 containing DNA binding domain (DBD), oligomerisation domain (OD), a second transactivation domain (TA2), sterile alpha motif (SAM), and a post-inhibitory domain (PID). Figure adapted from Murray-Zmijewski et al. (2006).

TAp63 and ΔNp63 have very different functions, with TAp63 playing a predominantly tumour suppressive role and ΔNp63 playing an oncogenic role (Su et al., 2013). Moreover ΔNp63 can actually antagonize TAp63, p73 and p53 and block their activity (Yang et al., 1998; Liefer et al., 2000; Ratovitski et al., 2001; Rocco et al., 2006; Marcel et al., 2012). In addition to the two subgroups, further subcategories are created as a result of alternative splicing on the 3' end which can generate α, β, γ, δ and ε isoforms. All of these isoforms contain the DNA binding domain and the oligomerization domain (Yang et al., 1998; Mangiulli et al., 2009). In addition, the α and β forms contain a second transactivation domain and the α forms contain a sterile alpha motif and a post-inhibitory domain, which respectively are required for protein-protein interactions and to mask the transactivation domain of TAp63α preventing its activation (Thanos & Bowie, 1999; Straub et al., 2010).

p63 is predominantly a nuclear protein, and thus is expressed in the nucleus in the basal cells of the epidermis and also in a variety of cancers, including squamous cell carcinomas (Di Como et al., 2002). Cytoplasmic p63, however, has also been detected in certain cancers, for example, in melanoma and prostate cancer (Dhillon et al., 2009; Matin et al., 2013).

1.4.3. Function

1.4.3.1. Embryonic tissue

p63 null mice are unable to survive long after birth due to severe dehydration and display truncated limbs and deformed craniofacial structures (Mills et al., 1999; Yang et al., 1999). One of the key functions of the skin is to control fluid loss from the body. The skin of these mice, however, was unable to differentiate leaving it in an unstratified state. Although initial studies using p63 null mice had reported similar phenotypes, their explanation as to the role of p63 in the developmental process of the skin differed and still remains a controversial topic (Mills et al., 1999; Yang et al., 1999). Subsequent studies have been performed to try to understand this difference.

Candi et al., (2006) created p63 null mice expressing either ΔNp63α or TAp63α to investigate the potentially diverse roles of the two promoter variants. From these data, and previous data from one of the initial mouse models, it was found that mice expressing ΔNp63α were able to develop a basal layer of skin while mice expressing TAp63α were unable to form the skin. ΔNp63 was expressed in the basal layers and was able to activate early differentiation

markers and TAp63 was expressed in the suprabasal layers and activated late stage markers (Figure 1.12). These data, and data from Laurikkala et al. (2006), suggest that ΔNp63α is key to the development and proliferation of the epidermis and that only subsequent TAp63α expression allows differentiation of the cell.

These data have, however, been criticsed by Koster et al. (2007) due to the inability of the researchers to recover the normal phenotype when expressing both ΔNp63α and TAp63α. Koster et al. (2004) had previously claimed their model demonstrated that the expression of TAp63 was the initial key stage of epidermal development. They showed that expression of only TAp63 in single layered lung epithelia was sufficient to initiate stratification and that overexpression of TAp63 prevented the differentiation of the cells. At present this is still a debated topic (Koster et al., 2007; Candi et al., 2008).

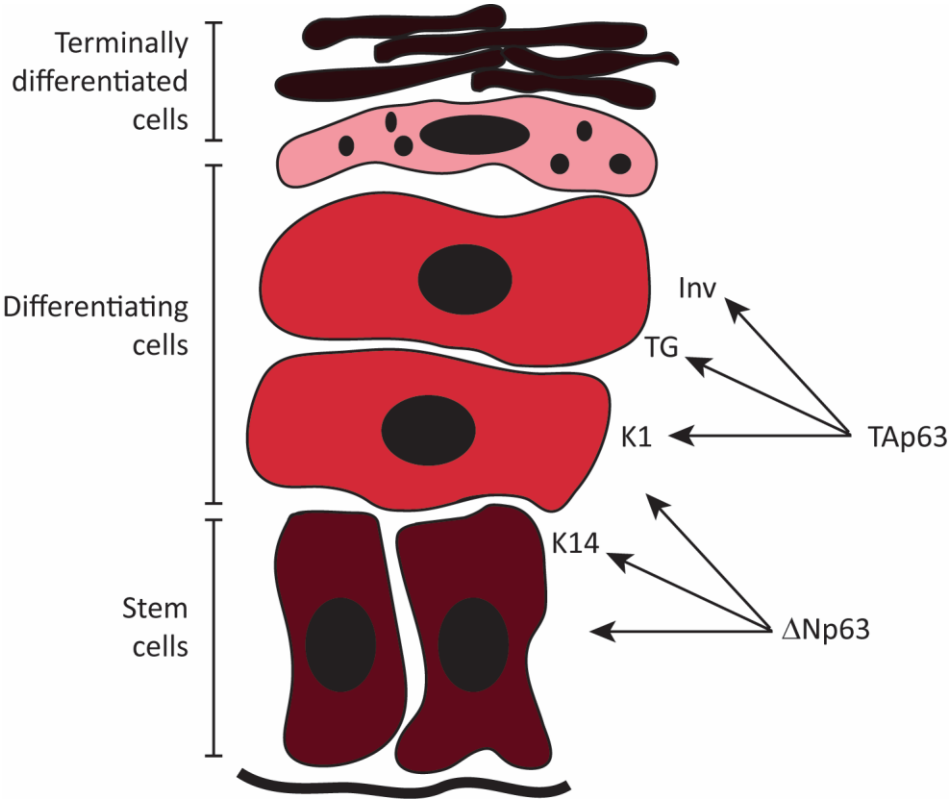


Figure 1.12. p63 and the development of the skin. ΔNp63 is expressed in the basal layers of the skin and is involved in the proliferation of the basal cells. TAp63 is expressed later and aids the expression of proteins involved in differentiation. Figure adapted from Candi et al. (2006).

1.4.3.2. Adult tissue

In adult tissue, ΔNp63 is the predominant isoform expressed in the mature epidermis and ensures its maintenance (Parsa et al., 1999). ΔNp63 is found in the basal cells of the adult epidermis and loses expression as the cells differentiate towards the surface. In cancer it is also ΔNp63 which behaves as an oncogene. In addition to cancer, p63 has been found to play a role in cellular senescence and ageing. Mice $p63^{+/-}$ display signs of accelerated ageing e.g., skin lesions, alopecia and severe degenerative disc disease of the spine and thus live for a shorter period of time (Flores et al., 2005; Keyes et al., 2005).

Further investigation into this phenomenon showed evidence for the involvement of both isoforms. TAp63 conditional knockout mice age prematurely, developing both blisters and skin ulcerations and also give rise to senescence of hair follicle-associated dermal and epidermal cells (Su et al., 2009). Supporting the notion of opposing roles ΔNp63α overexpression resulted in premature ageing correlating with decreased expression of Sirt1, a protein known to promote longevity in mice (Sommer et al., 2006).

1.4.4. Regulation

1.4.4.1. Post-translational modifications

Rapidly changing p63 protein levels are often observed in cell studies. These differences might be the result of post-translational modifications which affect protein stability and perhaps functionality of p63, although the mechanism of most of these modifications are not yet well defined.

The phosphorylation of TAp63 results in stabilisation of the protein. Several kinases have been implicated in the phosphorylation of TAp63. TAp63 can become phosphorylated when the cell is subjected to DNA damage, for example in cells subjected to cisplatin treatment, protein kinase c-abl, phosphorylates TAp63 stabilising the protein and activating the pro-apoptotic pathway (Gonfloni et al., 2009). TAp63γ can become phosphorylated by IKKβ and Plk1, preventing the apoptotic function of TAp63 in liver cancer cells (MacPartlin et al., 2008; Komatsu et al., 2009). In contrast, phosphorylation of ΔNp63 by ATM, CDK2 or p70s6K in HNSCC cells treated with cisplatin resulted in ΔNp63 becoming degraded (Huang et al., 2008). Similar findings were also reported by Westfall et al., (2005) who demonstrated that in cells which had undergone UV and paclitaxel treatment, ΔNp63 became phosphorylated and consequently degraded.

Other post-translational modifications leading to the degradation of p63 include ubiquitination, sumoylation and acetylation. Several studies have demonstrated a role for sumoylation in controlling ΔNp63 (Huang et al., 2004; Ghioni et al., 2002; Vivo et al., 2009). TAp63α contains a sumoylation site on the C-terminus and sumoylation of TAp63α has been shown to repress its transcriptional activity (Straub et al., 2010). TAp63 and ΔNp63 can become ubiquitinated and thus degraded by ITCH, an E3 ubiquitin ligase (Rossi et al., 2006). ΔNp63α can become targeted for degradation by the downregulation and destabilisation of scaffold proteins that can activate E3 ubiquitin ligases (Li et al., 2009).

1.4.4.2. ASPP family

As detailed in the previous section, it has recently been demonstrated that p63 was able to control iASPP at the protein and mRNA level in the stratified epithelia (Chikh et al*.*, 2011). Conversely, it was found that when the expression of iASPP was inhibited, the protein expression of p63 decreased but the mRNA levels were unaffected. It was discovered that this absence of change in p63 mRNA levels upon iASPP silencing was due to two microRNA, miR-720 and miR-574-3p. The microRNA expression increases when iASPP is silenced and, in turn, cause a decrease in the expression of p63, providing evidence for their involvement in inhibiting p63 translation (Figure 1.13) (Chikh et al*.*, 2011).

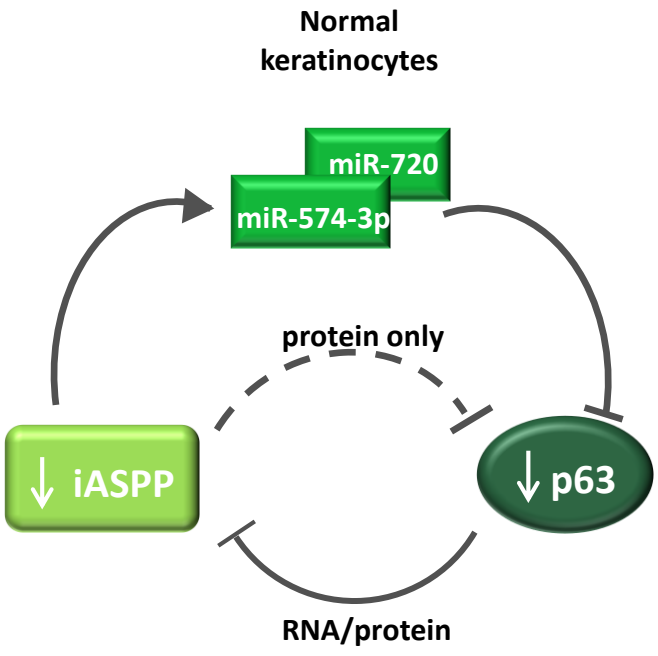


Figure 1.13. IASPP/p63 autoregulatory feedback loop. In the stratified epithelium p63 is regulated via iASPP and miR-574-3p and miR-720. p63 is able to regulate itself via the feedback loop, when p63 is expressed at high levels it promotes the expression of iASPP, which can in turn, inhibit the two microRNA able to control p63. When iASPP is silenced, the microRNA are able to bind to p63 and repress its translation. Figure adapted from Chikh et al. (2011).

Partially conflicting data came from Notari et al. (2011) however, using p53-null cell lines Saos-2 and H1299. These cells were transfected with ΔNp63 and TAp63 in the presence of exogenous iASPP. In these conditions cells exhibited 50% less transcriptional activity (using keratin 14 and envoplakin as a read out) than cells without exogenous iASPP, suggesting that iASPP binds p63 and inhibits its transcriptional activity (Notari et al., 2011). The expression levels of both ΔNp63 and TAp63 were unaffected in the presence of exogenous iASPP. This correlates with the finding that increased levels of p63 promote cellular senescence in MEFs, thus iASPP, an inhibitor of senescence/differentiation, may carry out this role via repressing the downstream activity of p63 (Notari et al., 2011).

Previous studies also had showed the ability of the other members of the ASPP family being able to bind to p63 (Robinson et al*.,* 2008). A recent study by Tordella et al. (2013) generated an SCC mouse model via an ASPP2 deletion. As mentioned previously, ASPP2 is a part of the ASPP family and has a similar structure to iASPP. The ASPP2 deletion inversely correlated with upregulation of p63. The data demonstrate that ASPP2 can repress p63 via NFκB pathway and thus can behave as a tumour suppressor in SCC via this route.

1.4.4.3. MicroRNA

Prior to the discovery of miR-574-3p and miR-720 controlling the expression of p63, Yi et al. (2008) generated a mouse model overexpressing miR-203 and found that the mice died at birth due to dehydration, a similar phenotype to that originally observed in p63 knockout mice (Mills et al., 1999; Yang et al., 1999). In the same year both Yi et al. (2008) and Lena et al. (2008) demonstrated that miR-203, located in the skin, was able to target and repress ΔNp63 thus inhibiting its stem cell maintenance and proliferative ability (Figure 1.14). In addition, Lena et al. (2008) showed that miR-203 could become upregulated upon genotoxic damage and regulate ΔNp63 in head and neck cancer. Later Scheel et al. (2009) discovered miR-302 was an important microRNA regulator of TAp63. It was shown that miR-302 was able to repress TAp63 in germ cells. In support of this model they found that upregulation of miR-302 in testicular cancer cells repressed TAp63 (Figure 1.14). Since these initial discoveries several additional microRNA have been found to regulate the different p63 isoforms. Table 1.2 lists some of these microRNA.

Table 1.2. Reported MicroRNA regulating p63.

MicroRNA	Isoform	Cell type	Study
miR-203	ΔNp63	Epithelia	Lena et al., 2008; Yi et al., 2008
miR-196a2	ΔNp63	Breast	Kim et al., 2013
miR-92	ΔNp63	Myeloid cells	Manni et al., 2009
miR-125b	ΔNp63	Hailey-Hailey keratinocytes; oral SCC	Manca et al., 2011; Boldrup et al., 2012
miR-130b	ΔNp63	Keratinocytes	Rivetti di Val Cervo et al., 2012
miR-720	ΔNp63	Keratinocytes	Chikh et al., 2011
miR-574-3p	ΔNp63	Keratinocytes	Chikh et al., 2011
miR-92a	TAp63	Acute promyelocytic leukaemia	Sharifi et al., 2014
miR-302	TAp63	Germ line	Scheel et al., 2009
miR-21	TAp63	Glioblastoma	Papagiannakopoulos et al., 2008; Quintavalle et al., 2012

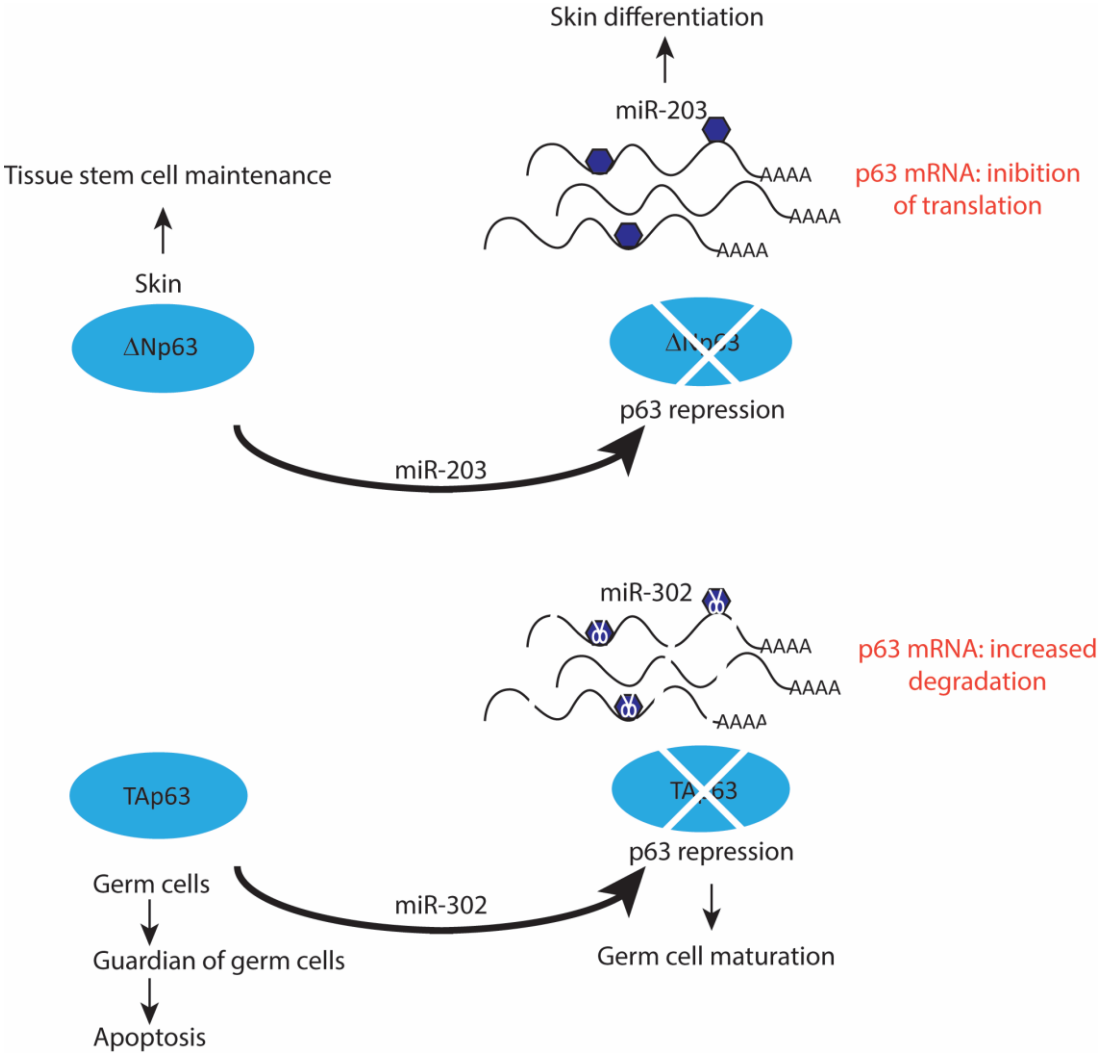


Figure 1.14. MicroRNA control of p63. miR-203 and miR-302 are able to control ΔNp63 and TAp63 respectively. miR-203 is able to repress ΔNp63 to promote skin differentiation and miR-302 is able to repress TAp63 and enable germ cell maturation. Figure adapted from Blandino & Moll (2009).

1.4.5. p63 and cancer

p63, although mutated in other disease types, such as ectodermal dysplasia and cleft lip/palate syndrome, is very rarely mutated in cancer, though a few mutations do exist (Rinne et al., 2007). Chromosome 3q27-28, where p63 is located, is often amplified in a range of cancers. Early studies, investigating a role for p63 in cancer, produced a lot of conflicting data. It is now known that this was due to antibodies being raised against the DNA binding

domain and thus were unable to discriminate between the different isoforms on p63. We now know that the TA and ΔN isoforms of p63 have different functions and this is clear in cancer. ΔNp63 is often found at high levels in cancer and thus plays an oncogenic role in the tumour, TAp63, on the other hand, can be found expressed at low levels in malignancies consistent with a tumour suppressor role.

TAp63 has been shown to behave in a similar fashion to the tumour suppressor p53. TAp63 has a broad range of transcriptional targets including cell cycle arrest gene *CDKN1A*, and apoptosis genes including *PUMA*, *NOXA* and *BAX* indicating its importance in the regulation of the cell and cancer (Su et al., 2013). TAp63 has also been shown to regulate dicer, a gene essential for microRNA synthesis (described in section 1.2) and miR-103b, a microRNA found to play a role in metastasis and is therefore potentially very important in regulating microRNA and metastasis (Su et al., 2010). In cancer, mutant p53 and TGFβ are able to bind to TAp63 and inhibit its anti-metastatic functions (Su et al., 2013). In bladder carcinoma and head and neck squamous cell carcinoma, a loss of TAp63 expression has been correlated with increased invasiveness of the tumour (Park et al., 2000; Su et al., 2010). In addition, TAp63 knockout mice developed metastatic tumour phenotypes (Su et al., 2010). In mouse models TAp63 behaves like a tumour suppressor gene by inhibiting the proliferation of adult stem cells and inducing senescence (Guo et al., 2009; Su et al., 2009).
In some instances however, TAp63 does not appear to be solely limited to playing a tumour suppressive role. A recent study into p63 and melanoma found that not only the ΔNp63 isoform but also TAp63 was able to confer chemoresistance in melanoma (Matin et al., 2013).

ΔNp63, on the other hand, can be found in keratinocytes with a high proliferative potential (Parsa et al., 1999). ΔNp63 has been shown to be upregulated in several cancers including squamous cell carcinomas. Crook et al. (2000) found high levels of ΔNp63 expression in nasopharyngeal carcinomas and Hibi et al. (2000) demonstrated high levels of ΔNp63 expression in both human head and neck, and lung SCC. An increasing number of studies are also providing evidence for ΔNp63 in cSCC when compared to normal skin (Senoo et al., 2001; Reis-Filho et al., 2002; Wrone et al., 2004; Dotto and Glusac, 2006; Alomari et al., 2014). In addition to this, p63 expression could potentially be used as a diagnostic marker for SCC (Lewis et al., 2005; Kargi et al., 2007; Khayyata et al., 2009; Ocque et al., 2011).

1.4.6. p63 therapeutics

As mentioned previously, targeted therapy towards p53 in cSCC is difficult due to the high mutation rate of p53. However, if by targeting tumour suppressor TAp63, we could reinstate some of the cellular pathways normally controlled by p53 this would prove advantageous (Bell & Ryan, 2008). Just like iASPP, p63 has a low mutation rate in tumours and therefore, from this perspective, could make an ideal target for therapy. The different isoforms of p63 that exist could also provide a greater scope for targets. For example, if decreasing or inhibiting ΔNp63 expression could prove too catastrophic for the body's normal cells, increasing TAp63 could provide a better alternative.

Zangen et al. (2005) found that upon cisplatin treatment in SCC cells, ΔNp63α became down regulated and proapoptotic protein TAp73 became stabilised. Encouragingly, this phenomenon appears only to occur in SCC cells and not in the normal epithelial cells (Deyoung et al., 2006). In addition, targeting down- or up-stream targets of these isoforms could be an alternative therapeutic angle. Inhibiting post-translational modifications of TAp63, for example, ubiquitination or sumoylation could increase the pro-apoptotic activity of TAp63 in the tumour (DeYoung & Ellisen 2007). Further investigation into understanding the role of p63 in the tumour could provide us with more targets to enable us to manipulate the expression of p63.

1.5. Hypothesis and aims

Recently, our group published a report demonstrating a link between iASPP and p63 via an autoregulatory feedback loop encompassing two novel microRNA, miR-574-3p and miR-720, in the stratified epithelia (Chikh et al., 2011). It was found that this feedback loop was essential for epithelial homeostasis as its effects were implicated in various cellular processes including adhesion, differentiation and proliferation. The aim of this research project is to dissect the role of iASPP, a novel crucial regulator of epidermal homeostasis, in keratinocyte skin carcinogenesis and to establish if the novel p63/iASPP feedback loop described is maintained or dysregulated in cSCC. Due to the importance of iASPP in maintaining cell homeostasis and the number of studies demonstrating an oncogenic role for iASPP in cancer, it is hypothesised that this feedback loop will be altered in cSCC.

The specific aims for this thesis are to explore:

1. The expression patterns and localisation of iASPP and p63 in cSCC
2. The integrity and regulation of iASPP/p63 autoregulatory feedback loop in cSCC
3. Pathophysiological effects of iASPP in cSCC

Chapter 2: Materials and Methods

2.1. Cell culture

HEK293, HaCaT and human primary fibroblast cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, UK) supplemented with 10% Foetal bovine serum (FBS, LabTech, UK), 2mM L-glutamine (Sigma, UK), 5000U/ml Penicillin and 5000µg/ml Streptomycin (pen/strep, Life Technologies, UK).

Cutaneous squamous cell carcinoma (cSCC) cell lines generated by the London Cancer Research UK Skin Tumour Laboratory and the N-TERT cell line were maintained in DMEM:F12 media at a 1:1 ratio (Life Technologies, UK) supplemented with 10% FBS, 2mM L-glutamine, 5000U/ml Penicillin, 5000µg/ml Streptomycin. An additional keratinocyte supplement was added to the media containing 5µg/ml transferrin (Sigma, UK), 0.4µg/ml hydrocortisone (Sigma, UK), 10^{-10}M cholera toxin (Sigma, UK), 5µg/ml insulin (Sigma, UK), $2x10^{-11}$M lyothyronine (Sigma, UK) and 10ng/ml epidermal growth factor (Serotec, UK).

All cell cultures were grown at 37°C in a 5% CO_2 atmosphere. Cell lines were passaged or pelleted upon reaching approximately 70% confluency. Prior to passaging, cells were washed in phosphate buffered saline (PBS, Life Technologies, UK) to remove any dead cells/cell debris and subsequently detached with trypsin:EDTA 1:1 (Life Technologies, UK), before being neutralised with media. Cells were then pelleted by centrifugation at 1200rpm for 5 min and either resuspended in media and reseeded, or frozen for experimental use at -80˚C. Cells required for freezing were trypsinised and pelleted as stated above before being resuspended in a solution of 90% FBS and 10% dimethyl sulphoxide (Sigma, UK). Cells were aliquoted in cryotubes and frozen at -80˚C before being stored long term in liquid nitrogen. To recover cells from frozen, cells required thawing at 37°C before being washed in media and subsequently resuspended and seeded in media. The media was then replaced after 24 h.

During differentiation assays, cells were maintained in EpiLife® medium (60µM Ca^{2+}) (Life Technologies, UK) and supplemented with Human Keratinocyte Growth Supplement (Life Technologies, UK). To stimulate terminal differentiation cells were treated with 2mM Ca^{2+} (Sigma, UK).

2.2. p63 plasmid preparation

p63 plasmids were kindly donated by G. Melino (MRC, Leicester). The pcDNA3.1 vector expressing an N-terminally HA-tagged protein was used. Chemically competent *Escherichia coli* cells (One shot® TOP10; Invitrogen™, USA) were transformed with 20ng DNA using a heat shock technique. Briefly, the DNA and plasmid were incubated together for 20 min on ice before being placed in a water bath at 37°C for 1 min then returned to ice. Following this 500µl Luria Bertani (LB) medium (Sigma, UK) was added and incubated at 37°C, shaking for 1h allowing expression of the antibiotic resistance marker. A 250µl aliquot of transformed cell suspension was spread evenly onto plates containing the appropriate antibiotic, in this case ampicillin. Plates were prepared by dissolving LB agar into ddH_20 and heating before adding ampicillin (150 µg/ml) to ensure selective bacterial growth. Plates were incubated overnight at 37°C. The following day individual colonies were picked from the plates and incubated with 4ml LB medium plus ampicillin (150 µg/ml) at 37°C on a shaker for 5h. Following this, an additional 200ml of LB medium (+ ampicillin 150 µg/ml) was added and left overnight on a shaker at 37°C. Cultures were then pelleted and the DNA was isolated using the Qiagen (UK) Plasmid Maxi Kit (High Speed) protocol. The DNA concentration was measured using a Nanodrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA).

2.3. Stable infection with short hairpin RNA (shRNA)

SMARTvector 2.0 Lentiviral shRNA particles were purchased from Dharmacon, GE Healthcare, UK. Three different constructs containing sequences targeting iASPP were tested (Table 2.1). The human cytomegalovirus (hCMV) promoter drives the expression of the gene targeting construct. Cells were transduced with shRNA lentiviral particles at a MOI 2 using polybrene (5µg/ml) (Merck Millipore, USA) to enhance the transduction of cells. The transduction efficiency was measured by assessing the percentage of cells expressing turboGFP after transduction. Cells were selected with puromycin (2.5µg/ml) due to a puromycin resistance gene present in the plasmid.

Table 2.1. ShRNA targeting sequences.		
Vector	**Gene target sequence**	**Source Clone ID**
hCMV-TurboGFP	GGAGTAAAGTCTAGCAGGA	SH-003815-01-10
hCMV-TurboGFP	AGTCGCTGGCCATGAAACA	SH-003815-02-10
hCMV-TurboGFP	AAGGACAACCTCACTAGCG	SH-003815-03-10

2.4. Short interfering RNA (siRNA)

Cells were silenced using transfection reagent DharmaFECT 1 (Dharmacon, GE Healthcare, UK) according to the manufacturer's instructions. A pool of 3 *Silencer*® siRNA sequences targeting exons 5, 6, 8 and 11 (Ambion, Life Technologies, UK) were used to silence p63 (Table 2.2). A pool of 4 ON-TARGET plus SMART pool (Dharmacon, GE Healthcare, UK) were used to silence iASPP (Table 2.2). The individual siRNA sequences were purchased and tested alone to ensure that pooling the siRNAs delivered the most effective knockdown. Non-targeting siRNA (Dharmacon, GE Healthcare, UK) was used as a control to ensure the targeted siRNA had no unspecific effects. Initial optimisation assays were performed using siGLO (Dharmacon, GE Healthcare, UK).

Table 2.2. SiRNA targeting sequences.		
Gene	**SiRNA ID**	**Gene target sequence**
p63	siRNA ID 4893	GGUUGGCACUGAAUUCACG
	siRNA ID 217144	CGUAUUCCACUGAACUGAA
	siRNA ID 217143	GCACACAAUUGAAACGUAC
iASPP	siRNA J-003815-05	AGUAAAGUCUAGCAGGAUA
	siRNA J-003815-06	GCACGGGUGUUGGCGGAAA
	siRNA J-003815-07	GCAGACGUCGAGCAGAGUA
	siRNA J-003815-08	UCGAGAAGUGCGACCCUUA

Briefly, siRNA at a concentration of 50nM, was incubated with 4µl DharmaFECT 1 in a 6-well plate containing pen/strep-free media. Optimal p63 knock down was observed at 48h and iASPP knock down at 72h.

2.5. Pre-miR™ miRNA Precursors & Anti-miR™ miRNA Inhibitor

Pre-miR miRNA precursors and anti-miR miRNA inhibitors (Ambion, Life Technologies, UK) were transfected into the cell using DharmaFECT 1 according to the manufacturer's instructions. Pre-miR and anti-miR negative controls were used to ensure there were no unspecific effects of the pre-miR precursors and anti-miR inhibitors respectively.

2.6. Total RNA extraction, first strand cDNA synthesis & quantitative PCR

Total RNA were extracted using the miRNeasy Mini Kit (Qiagen, UK) according to the manufacturer's instructions. The RNA concentration for each sample was measured using a Nanodrop ND-1000 spectrophotometer at an absorbance of 260nm (Thermo Fisher Scientific, USA). From the extracted RNA, 500ng was used to undergo first strand cDNA synthesis via the SuperScript® VILO™ cDNA synthesis kit (Life Technologies, UK), used according to the manufacturer's instructions. A PTC-225 Peltier thermal cycler (MJ Research, Canada) was programmed to 25˚C for 10 min, 42˚C for 60 min, followed by 85˚C for 5 min.

Quantitative-PCR reactions were performed by making a 20µl reaction mixture consisting of 50µg cDNA, 1µM of both forward and reverse primers and a 2X using Maxima SYBR® Green/ROX qPCR Master Mix (Thermo Fisher Scientific, USA) (Table 2.3). The reaction was run using a three-step protocol with the AB7500 Fast Realtime PCR System (Applied Biosystems, UK) (Table 2.4). All reactions were performed in triplicate on a 96-well plate and GUS, a housekeeping gene, was used to normalise the samples.

The comparative C_T method ($\Delta\Delta C_T$) was used to analyse the data. The average of the triplicate C_T values for the housekeeping control gene were subtracted from the average C_T values of the gene of interest = ΔC_T. The $\Delta\Delta C_T$ was calculated as the ΔC_T of the test sample minus the ΔC_T of the calibrator sample. The fold changes of expression were then calculated using the $2^{-\Delta\Delta C_T}$ formula (Livak and Schmittgen, 2001).

Table 2.3. Oligonucleotide primer sequences used for Q-PCR.				
Primer	**Sequence 5' – 3'**		**Annealing temp (˚C)**	**Reference**
	Forward	**Reverse**		
TAp63 (detects all isoforms of gene)	GGTGCGACAAACAAGATTGAG	GAAGGACACGTCGAAACTGTG	62	Chikh et al., 2011
ΔNp63 (detects all isoforms of gene)	GGAAAACAATGCCCAGACTC	GAAGGACACGTCGAAACTGTG	60	Chikh et al., 2011
iASPP	TCCTTTGAGGCTTCACCCTG	CGTGGATTTCCTCATCATCACCG	64	Chikh et al., 2011
GUS	AAACGATTGCAGGGTTTCAC	CTCTCGTCGGTGACTGTTCA	60	Matin et al., 2013
Zeb1	GTCCAAGAACCACCCTTGAA	TTTTTGGGCGGTGTAGAATC	60	Biddle et al., 2011
Zeb2	CGGTAGTGAGTCATAATGGT	GTCTCCTTGAGTCAGTAGTC	60	Unpublished data (Ankit Patel)
E-cadherin	GAACGCATTGCCACATACAC	AGCACCTTCCATGACAGACC	60	Biddle et al., 2011
Twist 1	GTCCGCAGTCTTACGAGGAG	CCAGCTTGAGGGTCTGAATC	60	Biddle et al., 2011
Vimentin	CCCTCACCTGTGAAGTGGAT	GACGAGCCATTTCCTCCTTC	60	Biddle et al., 2011

Table 2.4. Thermal cycling conditions for SYBR green qPCR.			
Step	**Temperature ˚C**	**Time**	**No. of cycles**
UDG pre-treatment	50	2 min	1
Initial denaturation	95	10 min	
Denaturation	95	15 s	40
Annealing	60-65 (depending upon primer used)	30 s	
Extension	72	30 s	

2.7. MicroRNA extraction, TaqMan® microRNA reverse transcription & quantitative PCR

MicroRNA extraction was carried out using the miRNeasy Mini Kit (Qiagen, UK) according to the manufacturer's instructions. The RNA concentration for each sample was measured using a Nanodrop ND-1000 spectrophotometer at an absorbance of 260nm. From the extracted RNA, 2ng was used to undergo reverse transcription using TaqMan® microRNA synthesis kit (Applied Biosystems, UK) according to the manufacturer's instructions. Primers were purchased from Ambion, Life Technologies, UK. The thermal cycler was programmed to 16˚C for 30 min, 42˚C for 30 min, followed by 85˚C for 5 min.

Quantitative PCR reactions were performed with the product from the reverse transcription reaction using the TaqMan MicroRNA Assay (Applied Biosystems, UK). Briefly, reverse transcription products were incubated along with a TaqMan microRNA probe and a TaqMan Universal PCR Master Mix. All reactions were performed in triplicate on a 96-well plate and RNU48, a housekeeping control, was used to normalise the samples. The reaction was run using an AB7500 Fast Realtime PCR System (Applied Biosystems, UK) (Table 2.5). The C_T values for the microRNA of interest were normalised to the C_T value for RNU48. The miRNA samples were analysed using the $\Delta\Delta C_T$ method as described in section 2.6.

Table 2.5. Thermal cycling conditions for TaqMan qPCR		
Temperature ˚C	**Time**	**No. of cycles**
95	10 min	1
95	15 s	40
60	60 s	

2.8. MicroRNA array

Total RNA was extracted from si-iASPP or non-targeting siRNA treated cSCC cell lines using miRNeasy Mini Kit (Qiagen, UK), as described previously. Samples were then sent to the Cancer Genomics Lab, Fondazione Edo ed Elvo Tempia, Italy. MiRNA expression profiling was carried out using the one-colour labelling method. Labelling, hybridization, washing and slide scanning were performed following manufacturers' protocols (Agilent Technologies, USA).

Briefly, 100ng of total RNA was dephosphorylated and denatured; then a ligation and labeling step with Cy3 performed. Samples were hybridised to oligonucleotide glass arrays with sequences representing probes of 2006 human microRNAs from the Sanger database v19 (Human miRNA 8x60K Microarray Version 19, Agilent Technologies, USA). After 20 h slides were washed and scanned using an Agilent C dual-laser microarray scanner. Images obtained were analysed using Feature Extraction software v10.5.

Raw data extracted from each image were processed using R statistical environment and the linear modelling for microarray analysis library. A filtering function was applied to select only 'well above background' features. Cy3 median signal was log transformed and background subtracted using the 'normexp' method, with an offset of 20. Between-array normalisation was applied, using the 'quantile' method and signals from replicated probes were averaged. For each cSCC cell line and each unique probe, the non-targeting control signal was subtracted from si-iASPP treated signal and a moderate *t*-test was applied to detect differentially expressed microRNAs. Slight modulation in terms of fold-change was accepted (+/- 1.25 FC) provided p-values were less than 0.01.

2.9. Luciferase reporter assay

The luciferase reporter assay was carried out to detect the effect of miR-211-5p-mediated, post-transcriptional regulation on p63. The luciferase assay requires a plasmid containing the 3' UTR of p63 and a plasmid containing a mutant 3' UTR as a control. The 3' UTR plasmid was already available in the lab but the mutant 3' UTR was generated, as it is required to be specific for each individual microRNA.

Mutagenic primers were designed using the guidelines supplied with the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, USA). Mutant 3' UTR plasmid was

generated using QuickChange II XL Site-Directed Mutagenesis Kit according to the manufacturer's instructions. Briefly, site-Directed Mutagenesis was carried out via thermal cycling combining a *PfuUltra* high fidelity DNA polymerase, mutagenic p63 primers and p63 plasmid. The reaction allows the primers to extend producing a mutant plasmid containing staggered nicks. Following this, the parental DNA template was digested using *Dpn* I endonuclease (specific for methylated and hemimethylated DNA – the plasmid DNA template was isolated from a dam^+ *Escherichia Coli* strain), selecting for the mutated DNA. The mutated DNA was then transformed into XL10-Gold ultracompletent cells and plated onto ampicillin containing agar plates. Mutant DNA was isolated using the Qiagen Plasmid Mini kit (Qiagen, UK). The DNA concentration was measured using a Nanodrop® ND-1000 Spectrophotometer.

HEK293 cells were seeded in triplicate in a 96 well plate at a density of 1.5 x 10^4 cells/well. The following vectors were used: psiCHECK™2-containing p63 3'UTR-firefly luciferase reporter and psiCHECK™2-containing mutant p63 3'UTR-firefly luciferase reporter. The following day cells were transfected with 10ng/µl plasmid using 30ng/µl polyethylenimine. Cells were left for 24h before being further transfected with Pre-miR as described in section 2.5. Cells were left for 48h. The Dual-Glo® Luciferase Assay System (Promega, USA) was used to determine the interaction between 3'UTR p63 and miR-211-5p, according to the manufacturer's instructions. The efficiency of transfection was normalised to Renilla luciferase activity.

2.10. *In situ* hybridisation of microRNA

Sections (5µm) were cut from paraffin tissue blocks obtained from Barts and the London NHS Trust (REC approval number 08/S1401/69) and sent to our collaborators at the University of Cologne, Germany. *In situ* hybridisation was carried out using the same technique described in Chikh at al. (2011). LNA probes of miR-574-3p and miR-720 oligonucleotides from Exiqon (Denmark) were labelled with DIG Oligonucleotide 3'-End Labelling Kit as described in the manufacturer's protocol (Roche Diagnostics, Germany). A control oligonucleotide from the Roche Kit was used as negative control.

Sections were deparaffinised in 100% xylene for 5 min followed by three incubations in 100%, 90% and 70% ethanol for 5 min before being washed in ddH_20. Sections were permeabilised

with 0.2% pepsin in 0.2M HCl for 15 min at 37°C. Sections were incubated twice in 0.1M glycine in PBS for 3 min at RT to stop pepsin digestion. Sections then underwent DNA-digestion by incubation with DNase 50U/ml for 1h at 37°C. Any endogenous peroxidase activity was inactivated by incubating sections in 3% H_2O_2 in methanol for 20 min at RT. Sections were washed twice for 3 min in ddH_2O then washed twice for 3 min in 2X saline sodium citrate (SSC; 0.3M NaCl, 0.03M $Na_3C_6H_5O_7$). Prehybridisation mix (50% formamide, 2X SSC, 0.05M Na_2HPO_4, 0.5% SDS, 1mM EDTA, 10mg salmon sperm DNA) was heated for 2 min at 95°C, applied to the sections and put on a heat plate at 90°C for 2 min. Slides were placed in a wet chamber for 1-2h at RT. The prehybridisation mix with 1pmol/50µl DIG labelled-probe was then applied to the sections. Slides were put on a heat plate (90°C) for 2 min, then put in a wet chamber and incubated at RT overnight. Slides were washed twice in 2X SSC for 15 min at RT, once in 1X SSC for 15 min at RT and once in 0.5X SSC for 15 min at RT.

Detection of DIG-labelled probes was done with anti-DIG-POD Fab fragments (Roche) in a 1:100 dilution and TSA Biotin System (Perkin-Elmer, USA) according to the manufacturer's protocol. All incubation steps were performed in a wet chamber. Sections were blocked with 0.5% blocking solution for 30 min. Anti-digoxigenin-POD Fab fragments (Roche, UK) were diluted 1:1000 and applied to the slides and incubated at RT for 30 min, followed by 3 washes with PBS, each 5 min. Amplification of the signal was carried out by diluting Biotinyl Tyramide Reagent 1:50 in Amplification Diluent and incubating for 20 min at RT, followed by three washes with PBS, each 5 min. Streptavidin-HRP was diluted 1:100 and incubated with the slides for 30 min at RT, followed by three washes in PBS, each 5 min. 3,3'-Diaminobenzidine (DAB) staining was carried out using a DAB substrate Kit for peroxidase (Vector Laboratories, UK). Sections were counterstained with haematoxylin and coverslips were applied to slides with DePeX (Serva, Germany). Images were taken on Nikon Eclipse 80i (Japan).

2.11. Protein extraction – NP40 lysis buffer

Cells were pelleted and washed in PBS prior to undergoing protein extraction with ice-cold lysis buffer (1M tris, 2.5M NaCl, 10% glycerol, 0.5M glycerophosphate, 1% Tween 20, 0.5% nonidet P40) containing 1X EDTA-free Complete Protease Inhibitor (Roche, UK) for 10 min. Cells were subjected to rapid freeze/thawing between dry ice and a 37˚C water bath before being vortexed and centrifuged at 13,000 rpm for 5 min. The supernatant was transferred to

a new Eppendorf and the protein concentration was measured using the Bradford assay according to the manufacturer's instructions (BioRad, USA). The Bradford assay works by producing a coloured compound directly proportional to the amount of protein present in the sample, the protein concentration was then analysed at a wavelength of 595nm on a Synergy HT Multi-Mode Microplate Reader (Bio-Tek, USA). The protein concentrations are determined from a bovine serum albumin (BSA) standard curve, formed using BSA concentrations between 0-10µg (x-axis) and the absorbance readings (y-axis). Protein samples were then boiled at 95˚C for 5 min with 1X NuPage® LDS Loading Buffer (Invitrogen™, USA) to denature the protein.

2.12. Protein extraction – urea lysis buffer

Both attached and floating cells were pelleted and washed in PBS prior to undergoing protein extraction with urea buffer (8M urea, 1M thiourea, 0.5% CHAPS, 50mM DTT and 24mM spermine) (Morris et al., 2014). The higher molecular weight iASPP band is mainly present in mitotic cells that may be found floating in the media. Protein concentrations were determined as described in section 2.11.

2.13. Protein extraction - subcellular fractionation

Extraction of cytoplasmic and nuclear proteins was performed using a NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. Briefly, cells were pelleted before being treated with Cytoplasmic Extraction Reagent I, after 10 min on ice Cytoplasmic Extraction Reagent II was added. Cells were vortexed and centrifuged for 5 min at maximum 13000 rpm. Immediately following this the supernatant (cytoplasmic extract) was transferred to a clean tube and kept at -80 ˚C until required. The insoluble fraction was suspended with Nuclear Extraction Reagent and incubated on ice for 40 min. Tubes were vortexed every 10 min within this period then centrifuged at 13000 rpm for 10 min. The supernatant (nuclear extract) was transferred to a clean tube and kept at -80 ˚C until required. Protein concentrations were determined as described in section 2.11. To ensure the specificity of the kit GAPDH and Lamin A, cytoplasmic and nuclear markers, respectively, were used.

2.14. Western blotting

Protein samples were run on SDS-PAGE gels comprising of a stacking gel and a resolving gel (Table 2.6 & 2.7). The percentage of resolving gel was modified depending upon the molecular weight of the protein of interest. iASPP and p63 were run on a 10 % gel. Typically 20-40µg of protein was loaded into each well. A Benchmark Protein marker (Invitrogen™, USA) was used as a reference for the molecular weight of the protein of interest. Gels were run in a Mighty Small Electrophoresis Unit (Hoefer, USA) with a 1X SDS-PAGE running buffer (250mM tris, 1.92M glycine, 1% SDS) at 150V until the proteins had fully resolved.

Table 2.6. Stacking gel (3 ml)	
Components	**Volume (ml)**
30% acrylamide solution	0.5
1M Tris pH 6.8	0.38
10% sodium dodecyl sulphate	0.03
10% ammonium persulphate	0.03
Tetramethylethylenediamine	0.003
ddH_2O	2.1

Table 2.7. 10% resolving gel (10 ml)	
Components	**Volume (ml)**
30% acrylamide solution	1.7
1M Tris pH 8.8	1.3
10% sodium dodecyl sulphate	0.05
10% ammonium persulphate	0.05
Tetramethylethylenediamine	0.002
ddH_2O	1.9

Once the running process was complete the gel was transferred onto a cassette containing 1 sponge, 2 pieces of blotting paper and a nitrocellulose transfer membrane (Amersham, Protran, GE Healthcare, UK), all pre-soaked in 1X transfer buffer (250mM Tris, 1.92M glycine, 20% methanol). Two further pieces of pre-soaked blotting paper and a pre-soaked sponge with placed on top of the gel to form a complete sandwich. The cassette was then positioned

in a Mini-PROTEAN® (BioRad, USA) transfer tank containing 1X transfer buffer and run for 2h at 350mA.

Once the transfer had completed, membranes were blocked in PBS containing 0.1% Tween and 5% milk for 1h on a rocker at RT to eliminate any unspecific binding of the antibody. After blocking, the membrane was incubated with the appropriate primary antibody diluted in PBS containing 0.1% Tween and 5% milk on a rocker overnight at 4˚C (Table 2.8).

The membrane was then washed 3 times for 5 min in PBS/Tween 0.1% before being incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (DAKO, USA) at a dilution of 1:10,000 in PBS containing 0.1% Tween and 5% milk for 1h on a rocker at RT. Membranes were then subjected to three further 5 min washes with PBS/Tween 0.1% before the secondary antibody was detected by adding ECL Plus (GE Healthcare, UK) to the membrane for 1 min and exposed to autoradiography film (Santa Cruz, USA). Films were developed with an automated developing machine. Densitometry analysis was carried out on the films using Image J.

Table 2.8. Primary antibodies.				
Antibody (species and type)	**Manufacturer and product Code**	**Western blot dilution**	**Immunocyto-chemistry dilution**	**Immunohisto-chemistry dilution**
iASPP (Mouse monoclonal)	Sigma A4605	1:1000	1:400	-
iASPP (Rabbit polyclonal)	Abcam ab34898	-	-	1:300
p63 H137 (Rabbit polyclonal)	Santa Cruz sc-8343	1:300	1:150	1:150
GAPDH (Rabbit polyclonal)	Abcam ab9485	1:2500	-	-
Cyclin D2 (Rabbit polyclonal)	Cell signalling #3741	1:500	-	-
Lamin A (Rabbit polyclonal)	Cell signalling #2032	1:500	-	-
Involucrin (Mouse monoclonal)	Abcam ab68	1:500	-	-
Zeb1 (Rabbit polyclonal)	Atlas Antibodies HPA027524	1:500	-	-
p53 DO-1 (Mouse monoclonal)	Santa Cruz sc-126	1:500	-	-
LC3 (Rabbit polyclonal)	Cell signalling #2275	1:500	-	-

2.15. Immunohistochemistry

Paraffin-embedded tissue samples were obtained from Barts and the London NHS Trust (REC approval number 08/S1401/69). Tissue samples were fixed in 10% formal saline solution before being embedded in paraffin wax. Upon use, tissue blocks were cut by a microtome at approximately 3-4 microns and mounted onto a positively charged slide. Sections were dewaxed in xylene twice for 3 min and rehydrated in alcohol twice for 3 min before being placed in running water for 5 min. Antigen retrieval was then performed. Sections to be stained for iASPP were microwaved in a vector antigen unmasking solution pH6 for 35 min. Sections to be stained for p63 were microwaved in Tris-EDTA unmasking solution pH9 for 35 min. Following antigen retrieval, sections were placed in running water for 5 min before any endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide solution for 15 min. Sections were washed three times in wash buffer before Normal Horse Serum (Vector R.T.U. kit, Vector Laboratories, UK) was applied for 20 min. The serum was then flicked off and the primary antibody was added at the appropriate concentration diluted

in antibody diluent (Table 2.8). Negative control sections were applied with antibody diluent without antibody. Sections were incubated at RT for 1h then washed three times in wash buffer. Universal Biotinylated Secondary Antibody (Vector R.T.U. kit, Vector Laboratories, UK) was added to the sections for 30 min, sections were washed in wash buffer twice then incubated with Vectastain Elite ABC Reagent (Vector R.T.U. kit, Vector Laboratories, UK) for 30 min. Sections were washed 3 times with wash buffer then incubated for 5 min with DAB solution before being washed in running water for 5 min. Slides were loaded into a machine staining rack, stained with haematoxylin then cover-slipped. Slides were scanned using a slide scanner (NanoZoomer 2.0-HT, Hamamatsu, Japan) situated in Core Pathology, Pathology Department, Royal London Hospital.

Scoring was carried out independently by myself and Consultant Pathologist, Dr Hasan Rizvi. Any discrepancies were resolved by a second round of analysis. A score index was obtained as a product of staining intensity (negative = 0; weak = 1; moderate = 2 and strong = 3) and percentage of positive tumour cell staining (negative = 0; 1-25% = 1; 26–50 % = 2; 51-75 % = 3 and 76-100% = 4) (Luo et al., 2012; Liu et al., 2012). The score index was carried out for both nuclear and cytoplasmic staining in both the tumour and any perilesional epidermis present. For statistical analysis a score of ≤4 was considered low expression and ≥6 as high expression.

2.16. Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (PFA) for 10 min followed by two 5 min washes in PBS. Cell membranes were permeabilised in 0.3% Triton X-100 for 2 min at RT. Coverslips were then washed twice with PBS for 10 min to remove any residual detergent. To avoid non-specific reaction, cells were incubated with 5% goat serum/PBS for 30 min. After removal of the serum, cells were incubated with primary antibody diluted in 5% goat serum/PBS overnight at 4°C (Table 2.8). Cells were washed 3 times for 10 min in PBS before being incubated with the appropriate fluorophore-conjugated secondary antibody (Dako, USA, diluted 1/500 in 5% PBS/goat serum) for 1 h at RT in the dark. Cells were washed twice for 10 min in PBS then incubated with 10µg/ml 4',6-diamidino-2-phenylindole (DAPI) for 10 min followed by two washes in PBS for 10 min. Coverslips were mounted onto a glass slide using Vectashield Mounting Medium (Vector Laboratories, USA) to prevent photobleaching

over time. Mounted slides were stored at 4˚C protected from light and viewed with a Zeiss upright 710 confocal (Germany).

2.17. Annexin-V binding assay

When cells undergo apoptosis, phosphatidylserine, usually located on the inner membrane of the cell becomes exposed on the surface of the cell. Annexin-V is able to bind to exposed phosphatidylserine and thus is used as a marker for apoptosis. To induce apoptosis, cells were treated with chemotherapeutic drugs etoposide and cisplatin for 18 h. After treatment, adherent cells and any floating cells in the supernatant were collected and pelleted before being washed in PBS. Cells were resuspended in 400µl 1X Becton Dickinson Annexin V Binding Buffer (BD Pharmingen™, USA). To this, 5µl Annexin-V-FTIC was added and tubes were incubated in the dark at RT for 15 min. After incubation, 200ng/ml DAPI was added to tubes as a marker for cell viability. Dead cells will take up DAPI more readily than live cells which will pump DAPI out. Cells were sorted at 10-20,000 events using a BD FACS Canto II Flow Cytometer (BD Biosciences, USA).

Results were analysed using the Becton Dickinson FACSDiva software version 6.1.03. Cells were classed as apoptotic if they were Annexin-V-FITC positive and DAPI positive or negative - right hand side quadrants of scatter plot.

2.18. Transwell cell migration assay

Cells were plated onto a 24 well plate containing Transwell® polycarbonate membrane cell culture inserts (Corning®, USA). The lower chamber of the wells contained 500µl of 10 % FBS-containing media and the upper chamber of the wells contained 200µl of 2% FBS-containing media, acting as a chemoattractant for the cells. Cells were seeded at a density of 1 x 10^4 cells in the 2% FBS-containing media and were incubated with the inserts for 24 h. After incubation, cells were washed once with PBS and fixed in 4% PFA for 15 min at RT. After fixing, wells were washed twice in PBS for 5 min before 0.1% (w/v) crystal violet was added to the wells and incubated for 4 min at RT. Wells were washed again before the non-migrated cells on the top of the membrane were removed with a cotton-wool bud. The migrated cells on the underside of the membrane were counted using a light microscope (Biddle et al, 2011).

2.19. Cell proliferation assay

Cells were seeded onto a 12 well plate on day 0 (10,000 cells per well). On days 3, 5 and 7 cells were detached and counted using a haemocytometer.

2.20. Colony forming assay

Cells were seeded at a density of 1000 cells per well on a 6-well plate and left for 10 days with a medium change every 3 days. After 10 days, cells were washed once with PBS and fixed in 4% PFA for 15 min at RT. After fixing, plates were washed twice in PBS for 5 min before crystal violet was added to the wells and incubated for 4 min at RT. Colonies of 50 cells or more were counted (Franken et al., 2006).

2.21. Organotypic culture

Organotypic cultures were produced using a combination of Collagen I, Rat Tail (Corning®, USA) and Matrigel® Basement Membrane Matrix (Corning®, USA) to mimic *in vitro* the epidermis of the skin (Nystrom et al, 2005). Organotypic gels were produced using 1:1 ratio of Type I collagen to Matrigel. Briefly, 7 volumes of Collagen:Matrigel were mixed on ice with 1 volume of 10X DMEM. The solution was neutralised with NaOH before 1 volume of FBS and 1 volume of $5x10^5$ human primary fibroblasts, resuspended in DMEM medium containing 10% FBS, were added. Millicell Hanging Cell Culture Inserts (polyethylene terephthalate, 0.4 µm, Merck Millipore, USA) were placed into a 12-well plate before 400µl of organotypic solution was added to each and left for 1h at 37˚C at 5% CO_2 to polymerise. cSCC cell lines were trypsinised and resuspended at a density of $5x10^5$ in 300µl. To ensure a confluent layer of cSCC cells covered the surface of the gel prior to air exposure, the cell suspension was added to the top of the gel and 1ml of medium was added to the bottom of the well. Inserts were incubated at 37˚C at 5% CO_2 for 24h. After incubation the medium on the top of the gel was aspirated creating a liquid/air interface for the cells. Culture medium was changed every 2 days and the organotypic cultures were maintained at 37˚C at 5% CO_2 for 10 days. Each experiment was performed in duplicate and repeated three times. Organotypic cultures were fixed for 1h in 4% PFA before being processed in an embedding machine. Slides were scanned using a slide scanner (NanoZoomer 2.0-HT, Hamamatsu, Japan) situated in Core Pathology, Pathology Department, Royal London Hospital. CellProfiler image analysis software was used to analyse the invasion index of cells.

2.22. Cell motility assay

Cells were seeded onto a 12-well plate at a density of $5x10^4$ cells/ml. The following day plates were placed into a CO_2 incubation chamber set to 37 °C. A Zeiss Axiovert 200M live cell imaging microscope (Germany) was used to take images of each well every 10 min for 8h. Metamorph Image Analysis Software was used to track the motility of the cells. Each different experimental condition was performed in duplicate and repeated three individual times. Three videos were taken per well, within those three images, 10 cells were tracked over 8h using the Metamorph Image Analysis Software.

2.23. Statistics

Microsoft Excel software was used to perform Student's *t*-tests. An unpaired, two tailed t-test was used to determine if two sets of data were significantly different from each other. A p-value of less than 0.05 was considered statistically significant.

A multivariate logistic regression was used to calculate odds ratios and 95% confidence intervals (95% CIs) using R version 3.0.2 stats library. A p-value of less than 0.05 was considered statistically significant.

Chapter 3: Expression patterns and localisation of iASPP and p63

3.1 Introduction and Aims

p63 is a master regulator of the skin. p63 null mice are unable to survive long after birth due to severe dehydration, a result of the skin being unable to differentiate (Mills et al., 1999; Yang et al., 1999). p63, although mutated in other disease types, such as ectodermal dysplasia and cleft lip/palate syndrome, is very rarely mutated in cancer, though a few mutations do exist (Rinne et al., 2007). Chromosome 3q27-28, where p63 is located, is often amplified in a range of cancers (Heselmeyer et al., 1996). Early studies investigating a role for p63 in cancer produced a lot of conflicting data. It is now known that this was due to antibodies being raised against the DNA binding domain. These antibodies were unable to discriminate between the different isoforms on p63. It is now known however that the TA and ΔN isoforms of p63 have different functions and this is clear in cancer.

In bladder carcinoma and head and neck SCC, loss of TAp63 expression has been correlated with increased invasiveness of the tumour (Park et al., 2000; Su et al., 2010). In mouse models TAp63 behaves like a tumour suppressor gene by inhibiting the proliferation of adult stem cells and inducing senescence (Guo et al., 2009; Su et al., 2009). In some instances however, TAp63 does not appear to be solely limited to playing a tumour suppressive role. A recent study from our group found both the ΔNp63 and TAp63 isoforms were able to confer chemoresistance in melanoma (Matin et al., 2013).

ΔNp63 which can be found in keratinocytes with a high proliferative potential lacks the N terminus and appears to have a dominant negative role on TAp63 and p53 inhibiting their activity and conferring an oncogenic phenotype (Yang et al., 1998; Parsa et al., 1999; Su et al., 2013). ΔNp63 has been shown to be upregulated in several cancers including SCC. Crook et al. (2000) found high levels of ΔNp63 expression in nasopharyngeal carcinomas and Hibi et al. (2000) demonstrated high levels of ΔNp63 expression in both human head and neck, and lung SCC. A handful of studies have also been performed demonstrating higher levels of ΔNp63 in cSCC when compared to normal skin (Senoo et al., 2001; Reis-Filho et al., 2002; Wrone et al., 2004; Dotto and Glusac, 2006; Alomari et al., 2014). So far, studies focussing on cSCC have been fairly limited in terms of sample size.

iASPP is largely expressed in epithelial cells and is a key regulator of epithelial homeostasis. Mouse models containing a deletion mutation in *PPP1R13L,* which encodes for iASPP, present an altered phenotype to wild-type mice with skin abnormalities (Herron et al., 2005; Toonen et al., 2012). iASPP has been well documented as an inhibitor of p53-induced apoptosis and thus is known to play an oncogenic role in the cell. It is therefore to be expected that iASPP would be involved in a cancer setting. Bergamaschi et al. (2003 and 2006) demonstrated that iASPP is upregulated in human breast carcinoma and similarly, Zhang et al. (2005) showed increased iASPP expression in acute leukaemia. Furthermore, several studies have linked iASPP to a number of other cancers including colorectal adenoma and carcinoma, leukaemia, hepatocellular carcinoma, prostate and ovarian cancers, non-small cell lung carcinoma, glioblastoma, endometrial endometrioid adenocarcinoma and melanoma (Saebo et al., 2006; Liu et al., 2009; Lu et al., 2010; Chen et al., 2010; Jiang et al., 2011; Zhang et al., 2011; Li et al., 2011; Liu et al., 2010; Lin et al., 2012; Lu et al., 2013). In addition to the above mentioned cancers, high iASPP expression has also recently been discovered in head and neck, and cervical SCC (Liu et al., 2012; Cao et al., 2013). Both of these studies have been carried out looking at SCC cell lines and SCC clinical samples. To the best of my knowledge no studies have been performed on iASPP in cSCC.

Due to the importance of iASPP and p63 in the skin and the oncogenic tendencies they have in cancer, I wanted to investigate iASPP and p63 in cSCC. Although several genes have been implicated in SCC pathogenesis the key molecular signalling pathway remain unclear. Investigating the role of iASPP and p63 in cSCC provides the potential for novel data on the molecular basis of squamous cell carcinogenesis.

Aims for this chapter:

1. Analyse the expression patterns of both iASPP and p63 in a panel of cSCC cell lines via qRT-PCR and western blot
2. Carry out immunohistochemical staining of iASPP and p63 in a range of cSCC tissue samples
3. Score and correlate iASPP and p63 staining in cSCC tissues with clinicopathologic characteristics of cSCC patients
4. Explore the cellular location of iASPP in cSCC

3.2 Results

3.2.1. High expression of iASPP and ΔNp63α found in a panel of cSCC cell lines

It has been well documented that both iASPP and p63 are upregulated in many cancers. The expression profile of iASPP in cSCC, however, has not yet been studied. In addition, p63 has undergone limited study in cSCC. To address this issue a panel of 10 human cSCC cell lines were analysed for both iASPP and p63 protein levels. cSCC cell lines were generated by the London Cancer Research UK Skin Tumour Laboratory (Table 3.1). Cell lines were generated from an equal number of immunocompetent and renal transplant patients. Each cell line had previously undergone targeted exome sequencing for p53, Notch 1 and 2, and Ras mutation. Six out of the ten cell lines had mutated p53 compared to seven containing Notch1 mutations, both of which are characteristics of cSCC (Brash et al., 1991; Forbes et al., 2010). An immortalised normal keratinocyte cell line (N-TERT) was included in the screening panel as a 'normal' control.

Table 3.1. cSCC cell lines.

Cell line	Differentiation Status	Patient status	Tp53 status	Notch 1 status	Notch2 status	NRAS status
PM2	Pre-malignant	Renal transplant	Mutant	Mutant	Mutant	Mutant
SCCIC4	Well	Immunocompetent	WT	WT	WT	WT
SCCT1	Well	Renal transplant	Mutant	Mutant	Mutant	WT
SCCIC15	Moderate	Immunocompetent	WT	Mutant	WT	WT
SCCIC18	Moderate	Immunocompetent	Mutant	Mutant	WT	WT
MET1	Moderate	Renal transplant	Mutant	Mutant	Mutant	WT
SCCIC1	Moderate	Immunocompetent	Mutant	Mutant	Mutant	WT
SCCT8	Poor	Renal transplant	Mutant	WT	Mutant	WT
SCCT11	Poor	Renal transplant	WT	WT	Mutant	WT
SCCIC8	Poor	Immunocompetent	WT	Mutant	WT	WT

WT: wild type

iASPP was initially discovered as a 477 amino acid protein but the full length of iASPP was later established as an 828 amino acid protein (Yang et al., 1999; Slee et al., 2004). The full length isoform of iASPP is deemed to be 100 KDa in size (Slee et al., 2004). Prior to investigation into cSCC cell lines, optimisation experiments were performed to ensure that the antibody targeted against iASPP recognised the full length isoform. Two antibodies were

chosen for investigation Sigma (A4605) and Abcam (ab34898). Sigma (A4605) provided the clearest signal and was chosen for further use.

p63 is found in several distinct isoforms. Preliminary experiments assessed which antibodies were able to recognise all the p63 isoforms and additionally contain no cross reactivity with p73, as has previously been documented with a popular p63 antibody, 4A4 (Rosenbluth et al., 2009). To aid the detection of the different p63 isoforms in cSCC, HEK293 cells were transfected with plasmids containing the different isoforms of p63; ΔNp63 α, β, γ and TAp63 α, β, γ. Cell lysates from transfected cells were run alongside a sample of lysates from cSCC cells as markers for p63 isoforms (Figure 3.1). Two different antibodies were tested that had no known p73 cross reactivity; p63 H129 and p63 H137 (Santa Cruz). The p63 H137 antibody detected all transfected isoforms of p63 and was selected for further study. Earlier research had shown ΔNp63 to be the predominant isoform expressed in cancer due to its oncogenic tendencies. In the small sample of cSCC cell lines I found the ΔNp63α isoform to have the strongest expression (Figure 3.1).

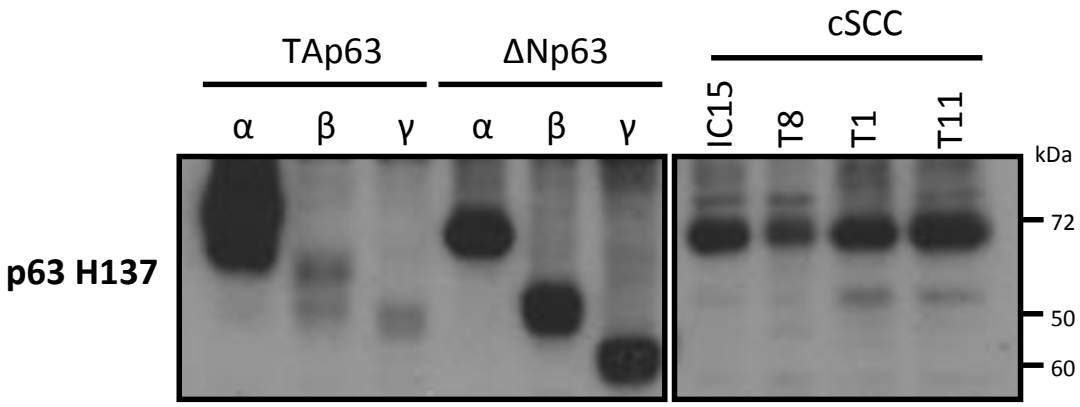


Figure 3.1. Expression of p63 isoforms. (A) Western blot with HEK293 cells transfected with p63 isoforms demonstrates the effectiveness of H137 p63 antibody alongside a sample of cSCC cell lines.

To ensure validity of western blot results, three different housekeeping proteins were tested to ensure no variance of levels between cell lines. Antibodies against housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH), beta-actin and alpha-tubulin were tested. GAPDH was chosen due to its stable expression in cSCC cell lines and also had the most appropriate molecular weight for the percentage gels in use.

Using the optimised antibodies and conditions, lysates from the panel of 10 cSCC cell lines along with lysate from the N-TERT cell line were run on a 10% SDS-PAGE gel and probed for p63 and iASPP (Figure 3.2A). The protein bands detected on the western blot films underwent densitometry analysis using Image J (Figure 3.2B). Western blot data illustrates a slight overall increase of iASPP protein expression in the cSCC cell line panel compared to N-TERT control. ΔNp63α protein expression data also shows a general increase in cSCC cell lines compared to N-TERT (IC8 p=0.0024). These data were in keeping with the predicted outcome due to the widespread publications on the oncogenic activity of iASPP and ΔNp63α.

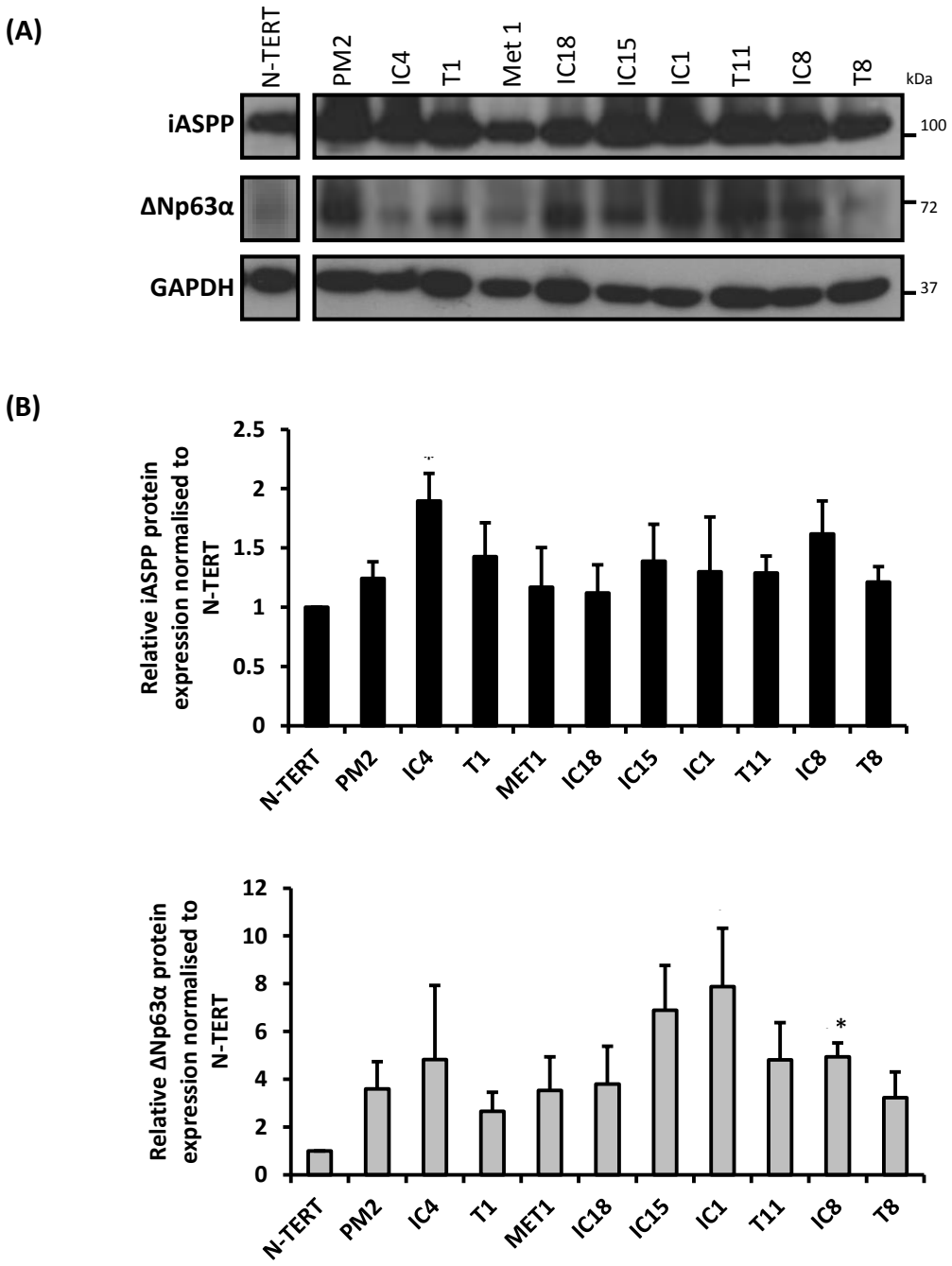


Figure 3.2. High expression of iASPP and ΔNp63α protein found in a panel of cSCC cell lines. (A) A panel of cSCC cell lines and a normal keratinocyte cell line (N-TERT) were screened for iASPP and ΔNp63α protein expression by western blotting. Cell lysates were run on a 10% SDS-PAGE gel and probed for iASPP and p63. GAPDH was used as a loading control. A representative western blot is shown. **(B)** Densitometry analysis showing the average iASPP and ΔNp63α protein expression levels when normalised to GAPDH and N-TERT. Error bars represent the SEM for three independent experiments. Statistical analysis was performed using a two-tailed, unpaired Students *t*-test. Experiments were corrected for multiple testing. P-value ≤ 0.005 = *.

Previous research has demonstrated a role for p63 in differentiation (Candi et al., 2006). iASPP is an inhibitor of differentiation and a promoter of proliferation (Chikh et al., 2011; Notari et al., 2011). For this reason it was interesting to correlate the differentiation status of the cells with iASPP and p63 protein expression. Poorly differentiated tumours tend to be more aggressive compared to well differentiated tumours. Although the levels of expression of both iASPP and ΔNp63α are variable between the cSCC cell lines, there appears to be no trend towards the differentiation status of the cell line and/or iASPP and ΔNp63α protein expression. It could be that there is no definitive pattern or another possibility could be that the cells, after being cultured from the patient, lose their differentiation profile. Western blotting with an antibody against involucrin, a molecular marker for keratinocytes differentiation, showed inconsistencies between the expression of involucrin and the apparent status of the cell (Figure 3.3) (Watt, 1983).

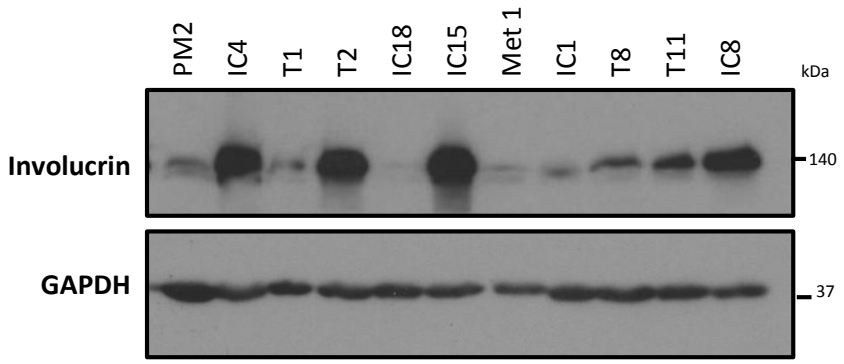


Figure 3.3. Differentiation profile of cSCC cell lines. Representative western blot of cSCC cell lines probed with involucrin. GAPDH was used as a loading control.

3.2.2. High iASPP and ΔNp63 mRNA levels in a panel of cSCC cell lines

In addition to checking the protein expression levels of iASPP and ΔNp63, mRNA levels were also studied. Primers previously used in work published by Chikh et al., (2011) were used for detecting mRNAs encoding ΔNp63 and the full length iASPP isoform. TAp63 primers were also used for initial tests but due to low mRNA levels only ΔNp63 was chosen for further study. RNA was extracted from lysates and reverse transcribed into cDNA before being analysed by qPCR. Three different housekeeping genes were tested to ensure consistency between the cell lines; beta-glucuronidase (GUS), actin and GAPDH. GUS was chosen to use as an internal control for all further experiments due to its stable expression in cSCC.

Figure 3.4 shows an overall increase in both iASPP and ΔNp63 mRNA levels compared to N-TERT. Two of the 10 cSCC cell lines tested showed significantly higher ΔNp63 levels compared to N-TERT (PM2 p=0.0004; Met1 p= 0.0045). But none of the cSCC cell lines showed significantly higher levels of iASPP compared to N-TERT. Interestingly, the pre-malignant cell line PM2 displayed significantly higher ΔNp63 levels compared to N-TERT correlating with the increase in ΔNp63 protein expression observed in Figure 3.2, hinting that perhaps amplification of p63 is an early event in SCC (Massion et al., 2003). iASPP protein levels in Figure 3.2 correlate with iASPP mRNA levels in Figure 3.4. p63 mRNA levels in Figure 3.4 show slight discrepancies compared to protein levels in Figure 3.2, however, the majority of cell lines follow the same pattern of expression. Additionally, there is a degree of variation among the cell lines and there appears to be no trend between the differentiation status of the cell line and/or iASPP and ΔNp63 mRNA levels. However, the above data are in keeping with the current research published on iASPP and ΔNp63 showing elevated levels in cancer.

It could perhaps be argued that the data in Figures 3.2 and 3.4 are limited due to the fact that both densitometry and qPCR cSCC cell line results are normalised to the N-TERT cell line. The N-TERT cell line is limited in its comparison with normal human primary keratinocytes (NHKs) due to the N-TERT cell line being immortalised by overexpressing the telomerase reverse transcriptase enzyme. NHKs contain telomeres on the ends of their chromosomes; these become shortened every time a cell divides, eventually leading to senescence of the cell. By expressing the telomerase reverse transcriptase enzyme in N-TERT cells, telomeres do not become shortened and thus are immortalised. Deletions of the *CDK2NA/INK4A* locus are also present in these cells (Dickson et al., 2000). These studies were originally performed using lysates collected from a range on NHKs and the cSCC cell line data were normalised to

these. Due to large result variation within the NHK group however, and differences in the expression of apparent housekeeping control genes between NHKs and cSCC cell lines, possibly due to the different conditions they require to grow, the N-TERTs were deemed a more suitable comparator.

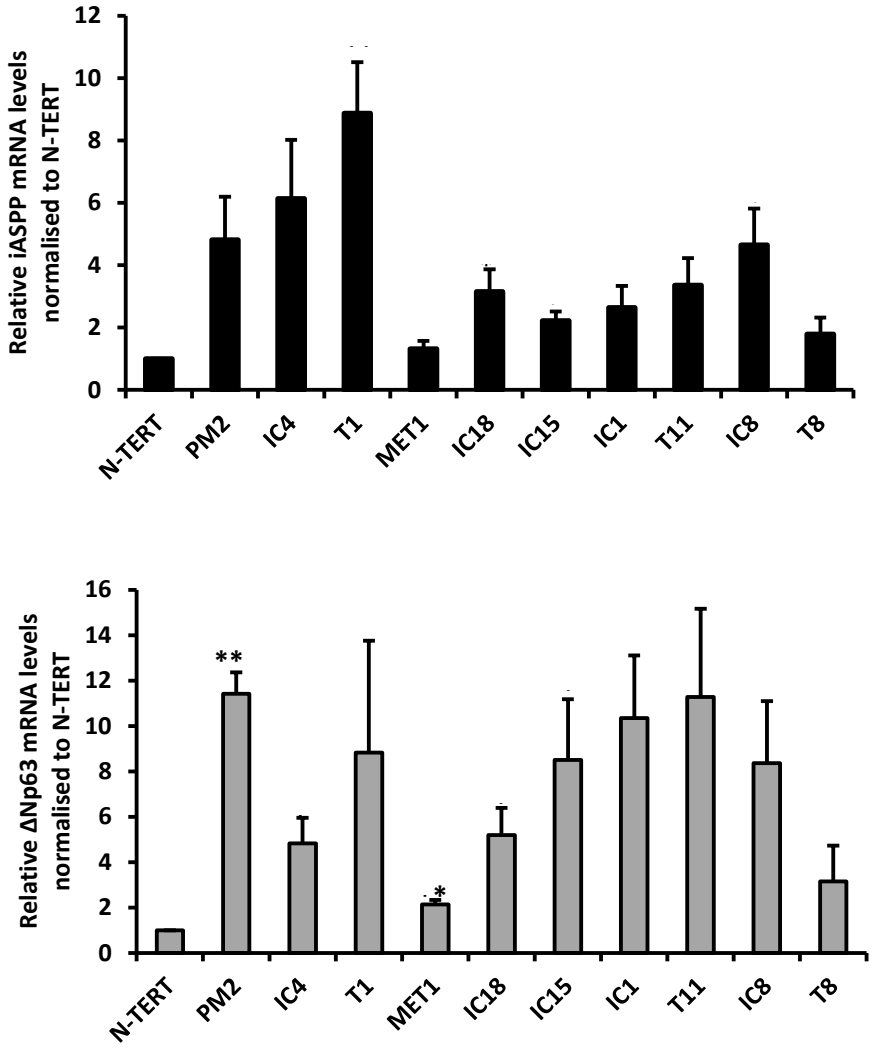


Figure 3.4. High levels of iASPP and ΔNp63 mRNA found in a panel of cSCC cell lines. A panel of cSCC cell lines and N-TERT were screened for iASPP and ΔNp63 mRNA by qRT-PCR. Housekeeping gene GUS was used as an internal control. mRNA levels of cSCC cell lines were normalised to N-TERT control. Error bars represent the SEM for three independent experiments performed in triplicate. Statistical analysis was performed using a two-tailed, unpaired Students *t*-test. Experiments were corrected for multiple testing. P-value ≤ 0.005 = *, ≤ 0.001 = **.

3.2.3. iASPP is highly expressed in human cSCC tissues

In order to validate the cell line data it was important to ensure the results were consistent in human tissue. A total of 106 cSCC tumours (well, moderately and poorly differentiated) were collected, stained and scored (Table 3.2). Tumours varied in terms of their clinicopathological characteristics providing a broad spectrum of samples. Normal skin sections were also included as a control. For iASPP staining, two antibodies were chosen for testing, Sigma (A4605) and Abcam (ab34898). Abcam (ab34898) had the least background staining and was chosen for further study. Several antigen retrieval conditions (pH of retrieval buffer, microwave versus waterbath and antigen retrieval time) were carried out to ensure specific and clean staining. Optimisation experiments were carried out in both normal skin and cervical sections where staining of iASPP had previously been published (Chikh et al., 2011; Notari et al., 2011).

Table 3.2. Clinicopathologic characteristics of cSCC patients.

Variables	Number of patients	Percentage of patients
Age:		
67	51	48.1
68	55	51.9
Sex:		
Female	33	31.1
Male	73	68.9
Tumour Site:		
Trunk	15	14.2
Upper limb	36	34.0
Lower limb	16	15.1
Head and neck (total):	39	36.8
Cheek	1	0.9
Forehead	5	4.7
Neck	4	3.8
Scalp	16	15.1
Temple	2	1.9
Ear/Nose/Lip/Eyelid	11	10.4
Differentiation Status:		
Well	52	49.1
Moderate	43	40.6
Poor	11	10.4
Immune Status:		
Renal transplant recipient	62	58.5
Immunocompetent	32	30.2
Other	12	11.3

Previous reports have described iASPP as a predominantly cytoplasmic protein; however, in the skin iASPP can be also expressed in the nucleus in the basal layer of the epidermis (Chikh et al., 2011; Notari et al., 2011). Consistent with this, the normal skin sections expressed predominantly nuclear iASPP (Figure 3.5A). A negative control containing no primary antibody was included along with a further control containing rabbit IgG to ensure no unspecific binding of the secondary antibody (Figure 3.5A).

Tumour sections showed variable nuclear and/or cytoplasmic iASPP staining (Figure 3.3B). To document this, both myself and consultant histopathologist, Hasan Rizvi, scored the sections based upon the intensity and percentage of cytoplasmic staining in both the tumour/perilesional epidermis versus the intensity and percentage of nuclear staining in both the tumour/perilesional epidermis. Scores were then combined; any discrepancies were resolved by a second round of consensus scoring. A score index was obtained as a product of staining intensity (negative = 0; weak = 1; moderate = 2 and strong = 3) and percentage of positive tumor cell staining (negative = 0; 1-25% = 1; 26–50 % = 2; 51-75 % = 3 and 76-100% = 4) (Luo et al., 2012; Liu et al., 2012). As the final immunoreactive score was calculated by multiplying the scores of staining intensity and the proportion of positive tumour cells, a score ≤4 was considered as patients with low expression and ≥6 as those with high expression (Table 3.3) (Luo et al., 2012; Liu et al., 2012). The scoring index obtained from each tumour was correlated with the clinicopathologic features of the patient (Table 3.3). The cut off of ≤67 and ≥68 years old was chosen as 67 years old was the median age of the patient sample. Statistical analysis was performed by Dr Ai Nagano at the Bioinformatics Unit, Barts Cancer Institute. A multivariate logistic regression was used to calculate odds ratios and 95% confidence intervals (95% CIs) using R version 3.0.2 stats library. Variables considered in the model were age, sex, tumour location, differentiation status and immune status. High odds ratio means high expression of nuclear iASPP or cytoplasmic iASPP. Although clearly a trend has emerged regarding the staining of nuclear and cytoplasmic iASPP and the differentiation status of the cell, the data are currently non-significant. The data comparing the differentiation status of the tumours is limited due to a low number of poorly differentiated samples available. Due to their relatively less common incidence, the tumour panel consists of only 11 poorly differentiated tumours compared to 43 moderate and 52 well differentiated tumours.

Graphs display the average staining index score (Figure 3.6). The scoring data show that in the normal epidermis, iASPP is nuclear in a high proportion of cells with relatively low

cytoplasmic staining (Figure 3.6A). In comparison, in the collective tumour samples there appears to be a switch with a high percentage of cells now expressing cytoplasmic iASPP and less cells expressing nuclear iASPP. The scoring data was also compared to the differentiation status of the tumour (Figure 3.6B and C). Data shows a trend in the differentiation status versus the scoring index of nuclear and cytoplasmic iASPP staining compared to the 'normal epidermis' taken from the same differentiation set. iASPP nuclear staining occurs at a higher percentage in the well differentiated tumours and decreases as the tumours become less well differentiated. The cytoplasmic staining follows the same pattern but inversely. It is also noteworthy that the patterns of iASPP localisation and differentiation are conflicting in the normal skin versus the tumour. In the normal skin iASPP is nuclear in the basal proliferating cells and becomes cytoplasmic upon differentiation in the suprabasal layers (Notari et al., 2011). In the tumour cells iASPP is highly nuclear in the well differentiated cells and less nuclear in the poorly differentiated cells.

Recent publications from both melanoma and prostate cancer show a strong association between nuclear iASPP expression and metastatic disease (Lu et al., 2013; Morris et al., 2014). Cao et al. (2013) found a strong correlation between high nuclear iASPP expression and increased chemoresistance and radioresistance in cervical cancer. Some researchers have speculated that the important role iASPP plays in interacting with nuclear based transcription factors e.g., p53, p63 and NF-κB, demonstrates that it is the nuclear form of iASPP that is the most active (Yang et al., 1999; Bergamaschi et al., 2003; Notari et al., 2011; Lu et al., 2013). In contrast, here I have shown that nuclear iASPP appears to be lost in the more aggressive tumour and that cytoplasmic iASPP is most prominent in the cSCC compared to normal skin. Support for the data from this project comes from publications showing high iASPP expression in the cytoplasm in a range of tumour types including HNSCC, ovarian cancer, cervical SCC, non-small cell lung cancer, hepatocellular carcinoma and conversely, prostate cancer (Chen et al., 2010; Lu et al., 2010; Zhang et al., 2011; Jiang et al., 2011; Liu et al., 2012; Cao et al., 2013). This conflicting data could suggest that the location/function of iASPP is cell type specific.

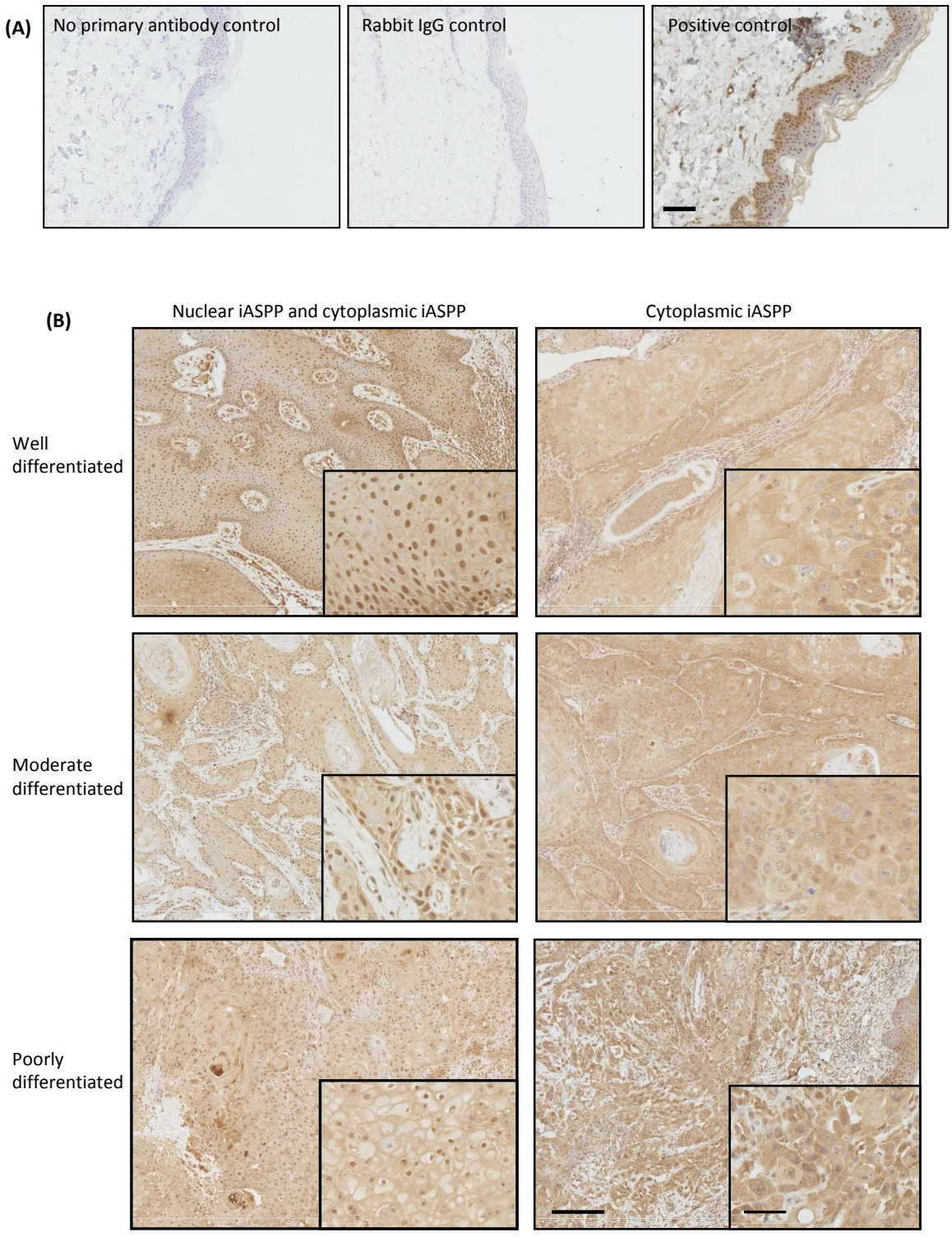
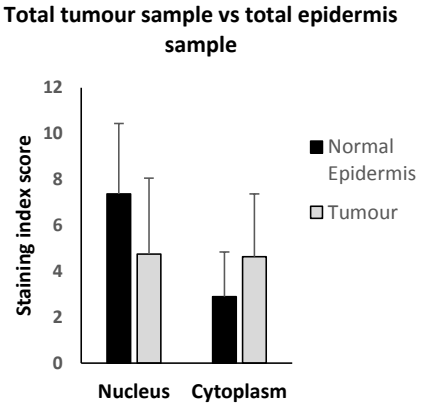


Figure 3.5. iASPP is highly expressed in human cSCC. (A) Immunohistochemical optimisation experiments for iASPP staining using normal human skin. Representative images of a negative no antibody control, negative rabbit IgG control and a positive control are shown. Brown staining represents iASPP, blue staining is negative. **(B)** Representative images of nuclear and cytoplasmic iASPP stained well, moderate and poorly differentiated cSCC tumours. Scale bar represents 200 µm, magnified image scale bar represents 50 µm.

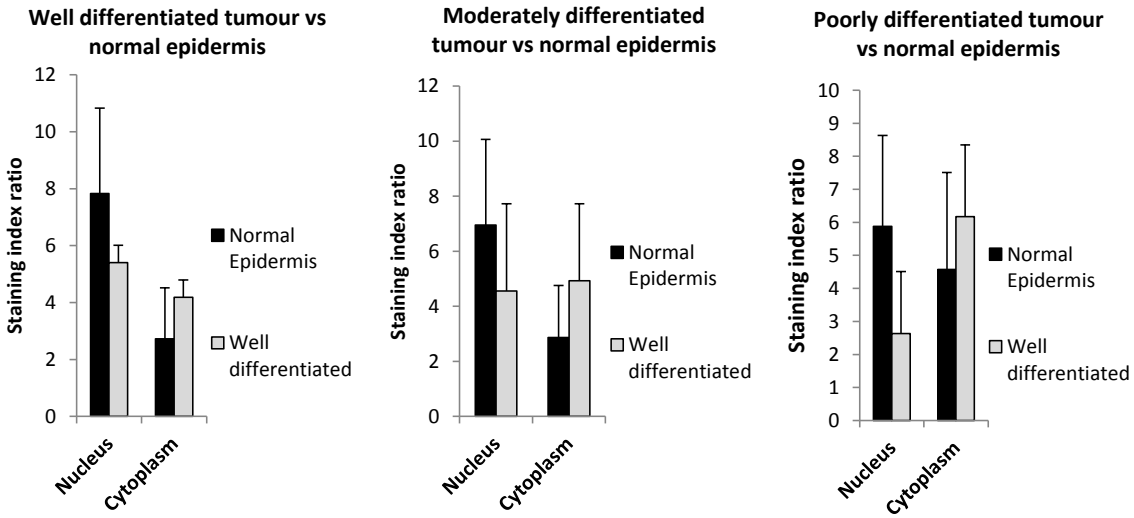
Table 3.3. Correlations between iASPP expression and clinicopathologic variables.

Variables	N	Nuclear iASPP expression						Cytoplasmic iASPP expression					
		Low (n)	High (n)	Low (%)	High (%)	Odds Ratio (95% CI)	p-value	Low (n)	High (n)	Low (%)	High (%)	Odds Ratio (95% CI)	p-value
Age													
≤ 67 years old	51	27	24	52.9	47.1	1 [Reference]		33	18	64.7	35.3	1 [Reference]	
≥ 68 years old	55	33	22	60	40	0.87 (0.33 - 2.26)	0.837	33	22	60	40	1.13 (0.41 - 3.10)	0.791
Sex													
Female	33	19	14	57.6	42.4	1 [Reference]		30	3	90.9	9.1	1 [Reference]	
Male	73	41	32	56.2	43.8	1.30 (0.51 - 3.35)	0.620	55	18	75.3	24.7	1.69 (0.62 - 4.97)	0.325
Tumour site													
Upper limb	36	17	19	47.2	52.8	1 [Reference]		25	11	69.4	30.6	1 [Reference]	
Lower limb	16	11	5	68.8	31.3	0.45 (0.12 - 1.62)	0.247	6	10	37.5	62.5	3.55 (0.97 - 14.04)	0.059
Head and neck	39	24	15	61.5	38.5	0.59 (0.21 - 1.67)	0.333	25	14	64.1	35.9	0.84 (0.27 - 2.53)	0.751
Trunk	15	8	7	53.3	46.7	0.92 (0.25 - 3.33)	0.860	10	5	66.7	33.3	0.79 (0.18 - 3.06)	0.716
Differentiation status													
Well	52	26	26	50	50	1 [Reference]		38	14	73.1	26.9	1 [Reference]	
Moderate	43	25	18	58.1	41.9	0.73 (0.29 - 1.82)	0.595	23	20	53.5	46.5	2.46 (0.94 - 6.70)	0.062
Poor	11	9	2	81.8	18.2	0.22 (0.03 - 1.02)	0.086	5	6	45.5	54.5	3.08 (0.73 - 13.81)	0.122
Immune Status													
RTR	62	34	28	54.8	45.2	1 [Reference]		39	23	62.9	37.1	1 [Reference]	
Immunocompetent	32	20	12	62.5	37.5	1.32 (0.43 - 4.12)	0.800	18	14	56.2	43.8	0.85 (0.26 - 2.68)	0.719
Other	12	7	5	58.3	41.7	0.91 (0.25 - 3.26)	0.901	9	3	75.0	25.0	0.84 (0.19 - 3.20)	0.887

(A)


Total tumour sample vs total epidermis sample
Staining index score
12
10
8
6
4
2
0
Nucleus
Cytoplasm
Normal Epidermis
Tumour

(B)


Well differentiated tumour vs normal epidermis
Staining index ratio
Normal Epidermis
Well differentiated
Nucleus
Cytoplasm
Moderately differentiated tumour vs normal epidermis
Staining index ratio
Normal Epidermis
Well differentiated
Nucleus
Cytoplasm
Poorly differentiated tumour vs normal epidermis
Staining index ratio
Normal Epidermis
Well differentiated
Nucleus
Cytoplasm

(C)

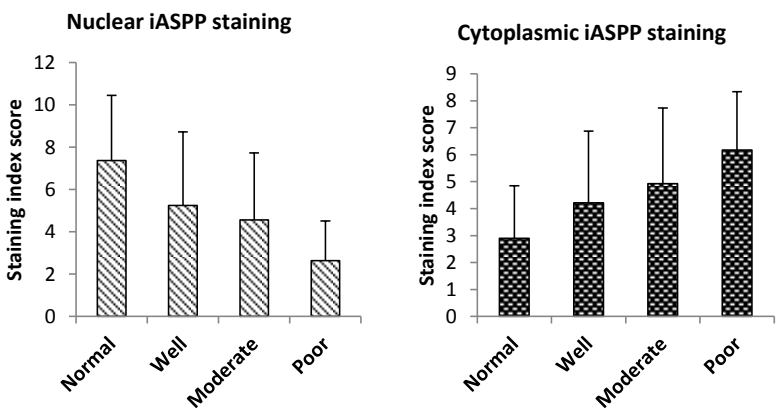

Nuclear iASPP staining
Staining index score
Normal
Well
Moderate
Poor
Cytoplasmic iASPP staining
Staining index score
Normal
Well
Moderate
Poor

Figure 3.6. A decrease of nuclear and increase of cytoplasmic iASPP staining is correlated the differentiation status of the tumour. **(A)** Graph displaying the average staining index score of nuclear and cytoplasmic iASPP in the total tumour sample versus total epidermal sample. **(B)** Graphs displaying average staining index score of nuclear and cytoplasmic iASPP in the well, moderate or poorly differentiated tumour sample versus total epidermal sample. **(C)** Graphs displaying average staining index score of both nuclear and cytoplasmic iASPP, respectively, compared to the differentiation status of the tumour. Error bars represent the standard deviation.

3.2.4 p63 is highly expressed in human cSCC tissues

p63 is a commonly used diagnostic marker for SCC (Lewis et al., 2005; Kargi et al., 2007; Khayyata et al., 2009; Ocque et al., 2011). Additionally, a couple of reports have also suggested that p63 can be used as a marker for poorly differentiated SCC (Kargi et al., 2007; Alomari et al., 2014).

The same panel of 107 tumours were stained for p63 (Table 3.4). An antibody was used that detects all p63 isoforms, Santa Cruz H137 (Figure 3.1). Considering the cell line data and previous literature, however, it is reasonable to assume that it is ΔNp63 that is being detected in the samples. Extracting protein from a microdissected SCC tumour, running the protein on a gel and western blotting with a p63 antibody to recognise all the isoforms could be a way to prove this. Optimisation experiments were carried out on normal skin sections where staining of p63 had previously been published (Figure 3.7A) (Reis-Filho et al., 2002; Di Como et al., 2002). In normal skin ΔNp63 is expressed in the basal layer of the epidermis. All stained sections were scored independently by myself and consultant histopathologist Hasan Rizvi. A score index was obtained as described in section 3.2.3.

p63 is predominantly a nuclear protein and is expressed in the nucleus in the basal cells of the epidermis and also in a variety of cancers, including SCC (Reis-Filho et al., 2002; Di Como et al., 2002). Cytoplasmic p63, however, has been detected in certain cancers, for example, in melanoma and prostate cancer (Dhillon et al., 2009; Matin et al., 2013). In this cSCC sample set sections positive for p63 showed a strong brown nuclear stain (Figure 3.7B). In contrast to iASPP, p63 stained sections were scored based upon the intensity of nuclear staining and the percentage of cells stained, no cytoplasmic p63 staining could be detected (Table 3.4). Both the tumour and any perilesional normal epidermis found within the same section were scored. The normal epidermis was used as an internal control to assess any difference between tumour and non-tumour cells. It is interesting to note, however, that as p63 is so highly expressed in the normal epidermis I was unable to see any additional increase of expression in the tumours, unlike in the previous cell line data. From the panel of tumours and normal skin sections stained for p63, all sections showed a high percentage of cells stained for nuclear p63 staining regardless of tumour versus non-tumour cells or tumour differentiation stage (Figure 3.8 A, B and C). These data do not support previous suggestions that p63 expression may be used as a marker of poorly differentiated SCC.

In the previous section I had described how iASPP displayed a predominantly cytoplasmic phenotype in the tumour compared to the perilesional epidermis. Chikh et al. (2011) had reported that in the normal epidermis iASPP and p63 were able to interact via an autoregulatory feedback loop. Data in the normal skin had shown a partial co-localisation of iASPP and p63. In this scenario, however, it appears that iASPP and p63 are no longer able to colocalise in cSCC, particularly in the moderate and poorly differentiated samples. Images analysed of both nuclear and iASPP staining from the same section of the tumour show a lack of co-localisation between iASPP and p63 suggesting that the autoregulatory feedback loop may be dysregulated in some SCC tumours (Figure 3.7B). These data are supported further by the staining cSCC tissues with fluorescently tagged secondary antibodies against both p63 and iASPP (Figure 3.9). Co-staining of iASPP and p63 in cSCC tumours shows the lack of co-localisation occurring within the tumour between iASPP and p63. p63 staining is found exclusively in the nucleus of the tumour cells and iASPP staining is predominantly cytoplasmic.

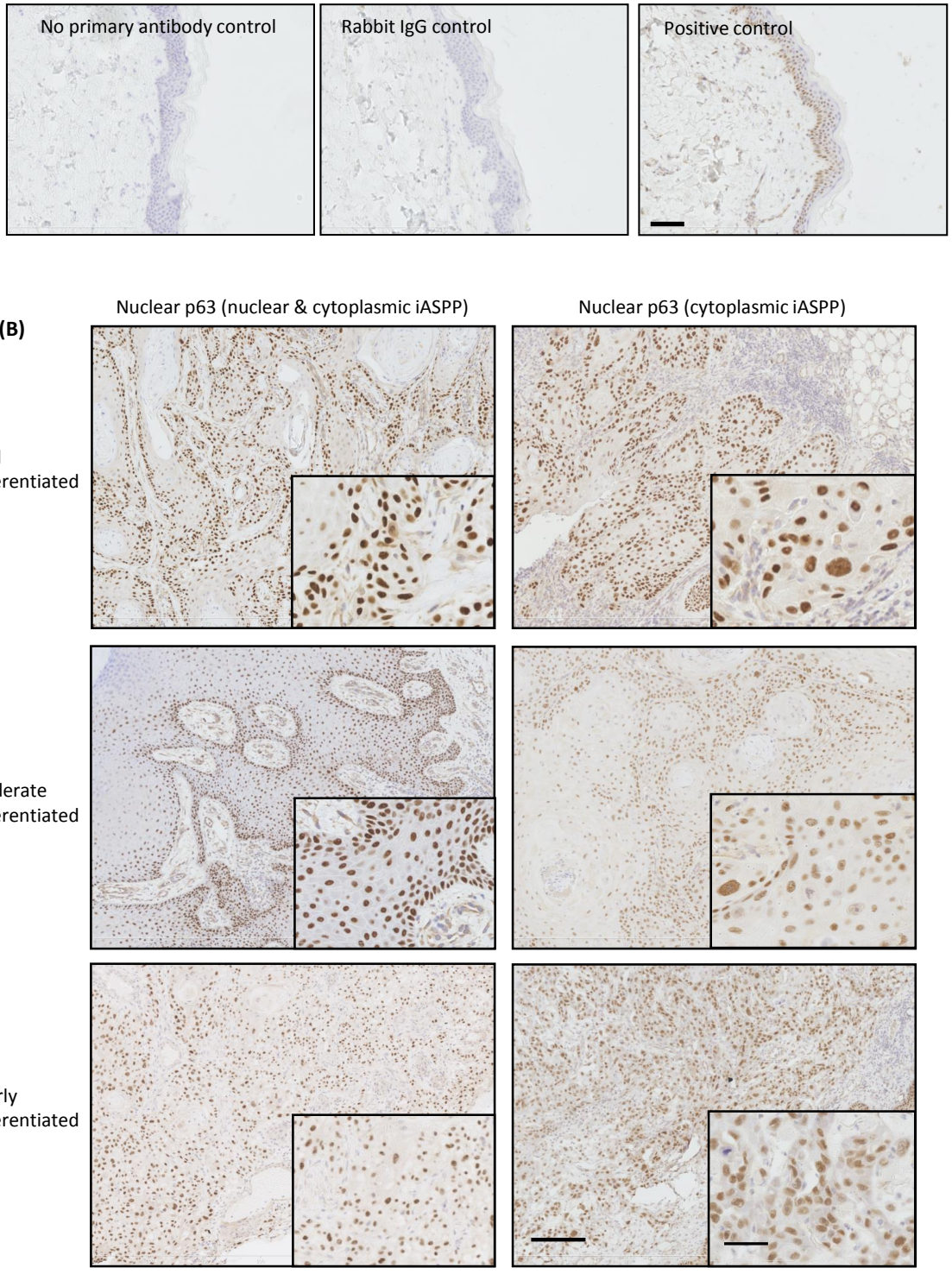


Figure 3.7. p63 is highly expressed in human cSCC. (A) Immunohistochemical optimisation experiments for p63 staining using normal human skin. Representative images of negative no antibody control, negative rabbit IgG control and a positive control are shown. Brown staining represents p63, blue staining is negative. **(B)** Representative images of p63 stained well, moderate and poorly differentiated cSCC tumours. Scale bar represents 200 µm, magnified image scale bar represents 50 µm.

Table 3.4. Correlations between p63 expression and clinicopathologic variables.

Variables	N	Nuclear p63 expression			
		Low (n)	High (n)	Low (%)	High (%)
Age					
≤ 67	51	0	51	0	100
≥ 68	56	0	56	0	100
Sex					
Female	34	0	34	0	100
Male	73	0	73	0	100
Tumour site					
Upper limb	37	0	37	0	100
Lower limb	16	0	16	0	100
Head and neck	39	0	39	0	100
Trunk	15	0	15	0	100
Differentiation status					
Well	52	0	52	0	100
Moderate	44	0	44	0	100
Poor	11	0	11	0	100
Immune Status					
Renal transplant recipient	62	0	62	0	100
Immunocompetent	33	0	33	0	100

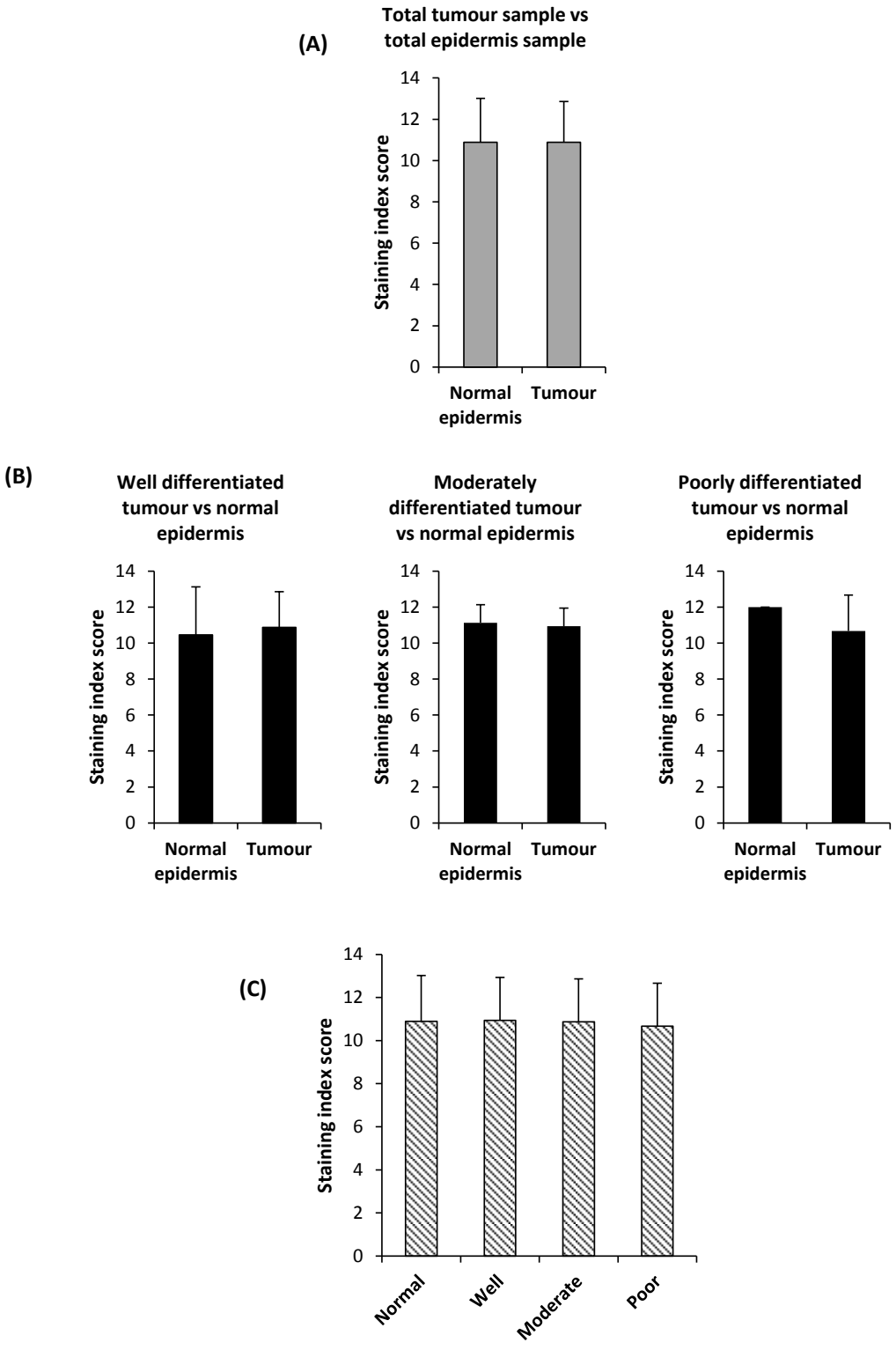


Figure 3.8. p63 expression is not correlated with differentiation status of tumour. (A) Graph displaying staining index score of p63 in the total tumour sample versus total epidermal sample. **(B)** Graphs displaying staining index score of p63 in well, moderate or poorly differentiated tumour sample versus total epidermal sample. **(C)** Graphs displaying a staining index score of p63 compared to the differentiation status of the tumour. Error bars represent the standard deviation.

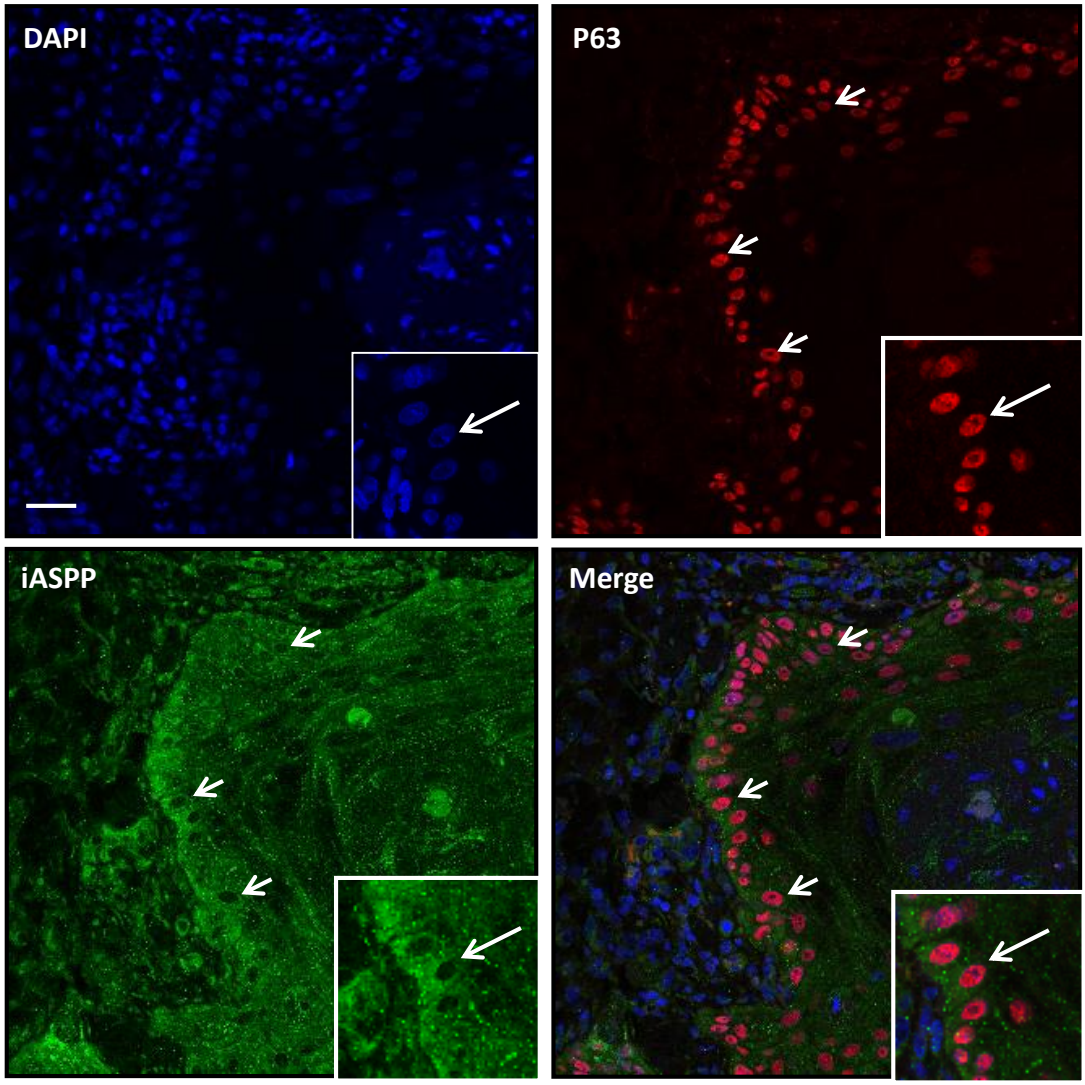


Figure 3.9. Localisation of iASPP and p63 in cSCC tumour. cSCC tumour immunostained for iASPP (green), p63 (red) and DAPI (blue). iASPP, p63 and DAPI images were merged. Scale bars represent 20µm.

3.2.5 Location of iASPP and ΔNp63α in a panel of cSCC cell lines

To explore whether the panel of cell lines screened in section 3.2.1 display the same phenotype as the tumour sample set, tissues were fixed and stained with immunofluorescence antibodies targeted against iASPP and p63 (Figure 3.10). As expected, p63 was consistently nuclear in all cell lines. The location of iASPP within the cell was also fairly consistent with the tissue data, with all of the cSCC cell lines largely expressing cytoplasmic iASPP. The only other major difference between tissue and cell line was the absence of iASPP cytoplasmic and nuclear variance between the differentiation status of the cell lines. This could be attributed to the potential discrepancies between the patients' tumour differentiation status and the differentiation status of the cell once cultured as mentioned earlier.

This data also complements the findings from section 3.2.4 that iASPP and p63 may no longer co-localise. The implications of this lack of co-localisation on the feedback loop are researched further in chapter 4.

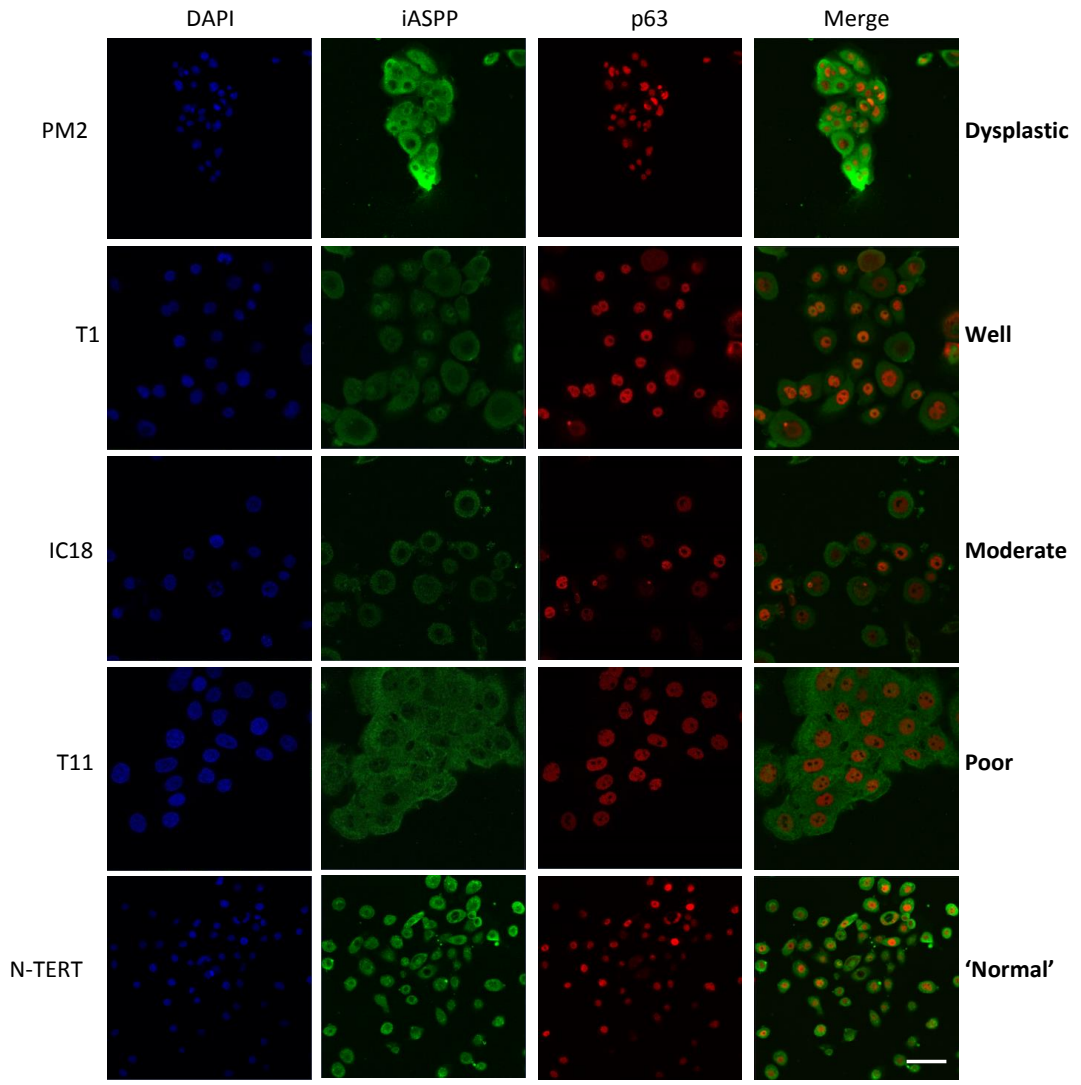


Figure 3.10. Localisation of iASPP and p63 in a panel of cSCC cell lines. A panel of cSCC cell lines and N-TERT were fixed and immunostained for iASPP (green), p63 (red) and DAPI (blue). iASPP and p63 images were merged.

Melanoma contains high levels of nuclear iASPP correlating with metastatic disease (Lu et al., 2013). Lu et al., (2013) have recently reported that in the cytoplasm, iASPP exists as an anti-parallel homodimer with the N-terminus interacting with the C-terminus. Phosphorylation of iASPP at S84/S113 by cyclin B1/CDK1 prevents iASPP residing in a homodimer structure and enables monomeric iASPP to shuttle to the nucleus, resulting in increased p53 binding and inhibition. Lu et al. (2013) suggest that nuclear iASPP is the active form of iASPP. Phosphorylated nuclear iASPP migrates slower on a SDS-PAGE gel than unphosporylated iASPP thus the existence of both nuclear and cytoplasmic iASPP in melanoma cell lines presents as a doublet band on a western blot.

In light of this publication and the volume of conflicting data supporting the importance of cytoplasmic versus nuclear iASPP expression, I wanted to determine whether phosphorylated iASPP was detectable in cSCC. I hypothesised due to my data and previous reports on nuclear/cytoplasmic iASPP that the function/location of iASPP may be cell type specific. Cytoplasmic iASPP was predominantly expressed in the more aggressive cSCC tumours and in all cSCC cell lines tested by immunofluorescence. It was predicted that there would be an absence of phosphorylated nuclear iASPP in cSCC. In the western blots in section 3.2.1 I was unable to detect an upper phosphorylated band. However, the protocol used was different from Lu et al. (2013). Using the same protocol from Lu et al. (2013) I still failed to see a second band in cSCC cell lines and N-TERT (Figure 3.11A). A melanoma cell line, Mel 505, was included as a positive control. Support for this data comes from Morris et al. (2014) who show that western blotting with HaCaT lysates also displayed little or no phosphorylated iASPP, suggesting a cell type specificity for this iASPP post-translational modification form.

To support these data, a subcellular fractionation was performed with N-TERT and several cSCC cell lines (Figure 3.11B). The cytoplasmic and nuclear fractions of the cells were separated and purified before being run on a gel and western blotted with an iASPP antibody. It is noteworthy here, that the nuclear iASPP detected is of the same molecular weight as the cytoplasmic iASPP, thus no upper phosphorylated band was identified as the nuclear fraction. This finding could either be as a result of different buffer use or suggest a cell type dependent phosphorylation of iASPP i.e., in cSCC iASPP may exist in the nucleus in an unphosphorylated form. Regardless of this, the data produced were in accordance with my hypothesis that in cSCC cells iASPP is expressed predominantly in the cytoplasm compared to the nucleus.

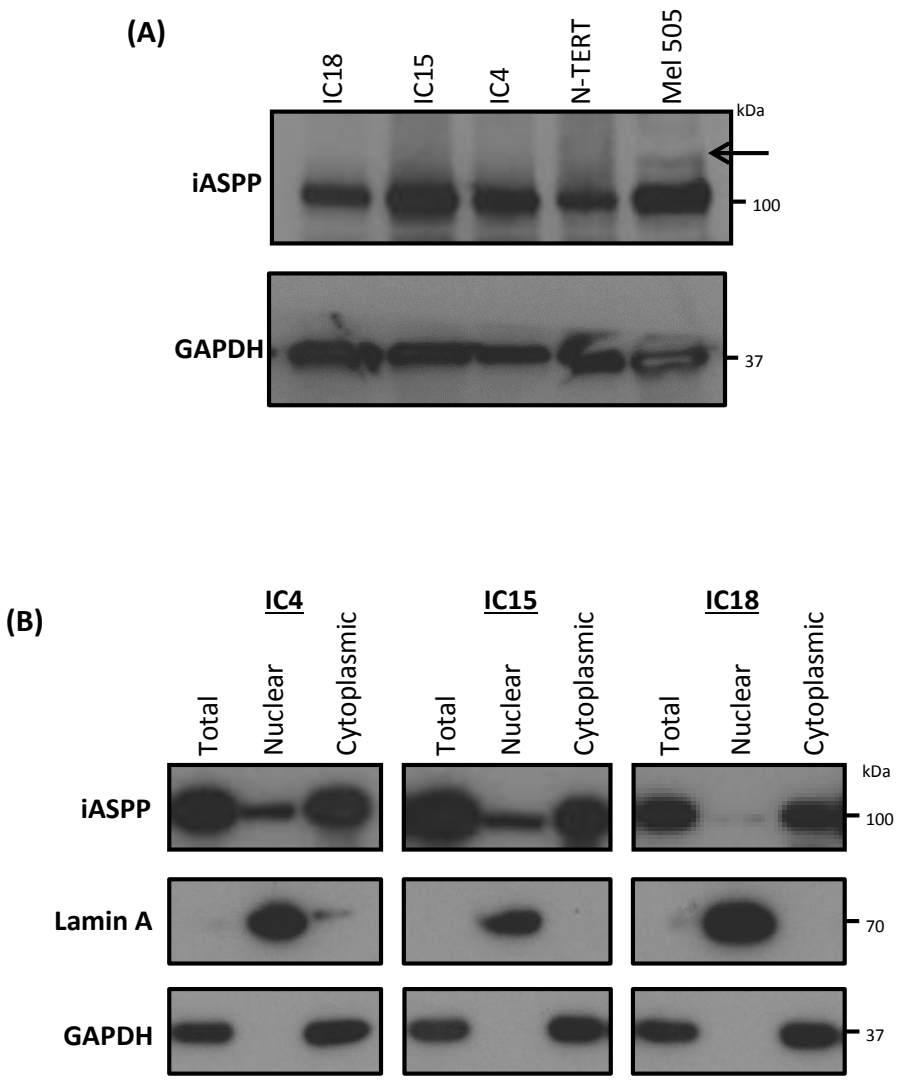


Figure 3.11. Localisation of iASPP and p63 in a panel of cSCC cell lines. (A) Three cSCC cell lines, N-TERT and melanoma cell line Mel505 lysed with urea buffer western blotted for iASPP. GAPDH was used as a loading control. Arrow points to phosphorylated iASPP. **(B)** Cytoplasmic (C) and nuclear (N) proteins were extracted from three cSCC cell lines. Extracts were run on a 10% SDS-PAGE gel and blotted for iASPP. Lamin A and GAPDH were used as controls for nuclear and cytoplasmic proteins respectively.

3.2.6 Calcium differentiation of cell lines alters the localisation of iASPP in cSCC cell lines

Data have shown that in the normal skin iASPP is both cytoplasmic and nuclear. cSCC tumour data shows that the less differentiated the tumour the less nuclear iASPP is and the more cytoplasmic it becomes. In cSCC cell lines iASPP is predominantly cytoplasmic. I therefore wanted to see if I could create a model whereby altered differentiation of the cells could produce a different iASPP phenotype. To do this I thawed cSCC cell lines in EpiLife Medium, with 60µM calcium supplemented with human keratinocyte growth supplement. In order to stimulate differentiation of the cell lines, the calcium concentration was increased to 2mM (Pillai et al., 1988). Aside from the morphological change in the cells, differentiation of the cell lines was assessed by measuring involucrin expression, a marker of keratinocyte terminal differentiation (Figure 3.12A) (Watt, 1983) with western blotting. Cells were either treated with 2mM Ca^{2+} or left untreated in EpiLife. Lysates collected from these cells underwent subcellular fractionation isolating both the nuclear and cytoplasmic fractions. Lysates were western blotted for iASPP. Lamin A and GAPDH were used as controls for nuclear and cytoplasmic proteins respectively. Densitometry analysis was performed using image J to quantify the western blots.

Chikh et al. (2011) found that upon terminal differentiation in normal keratinocytes, total iASPP expression decreased. Here I have shown using two cSCC cell lines, IC4 and IC15, that treatment of cells with 2mM Ca^{2+} slightly increased nuclear iASPP and marginally decreased cytoplasmic iASPP (Figure 3.12B). Although not significant this trend fits with the previous data in this project showing that the more differentiated the cell/tumour is the more nuclear iASPP is present. Reduction of nuclear iASPP correlates with the inability of cancer cells to completely differentiate.

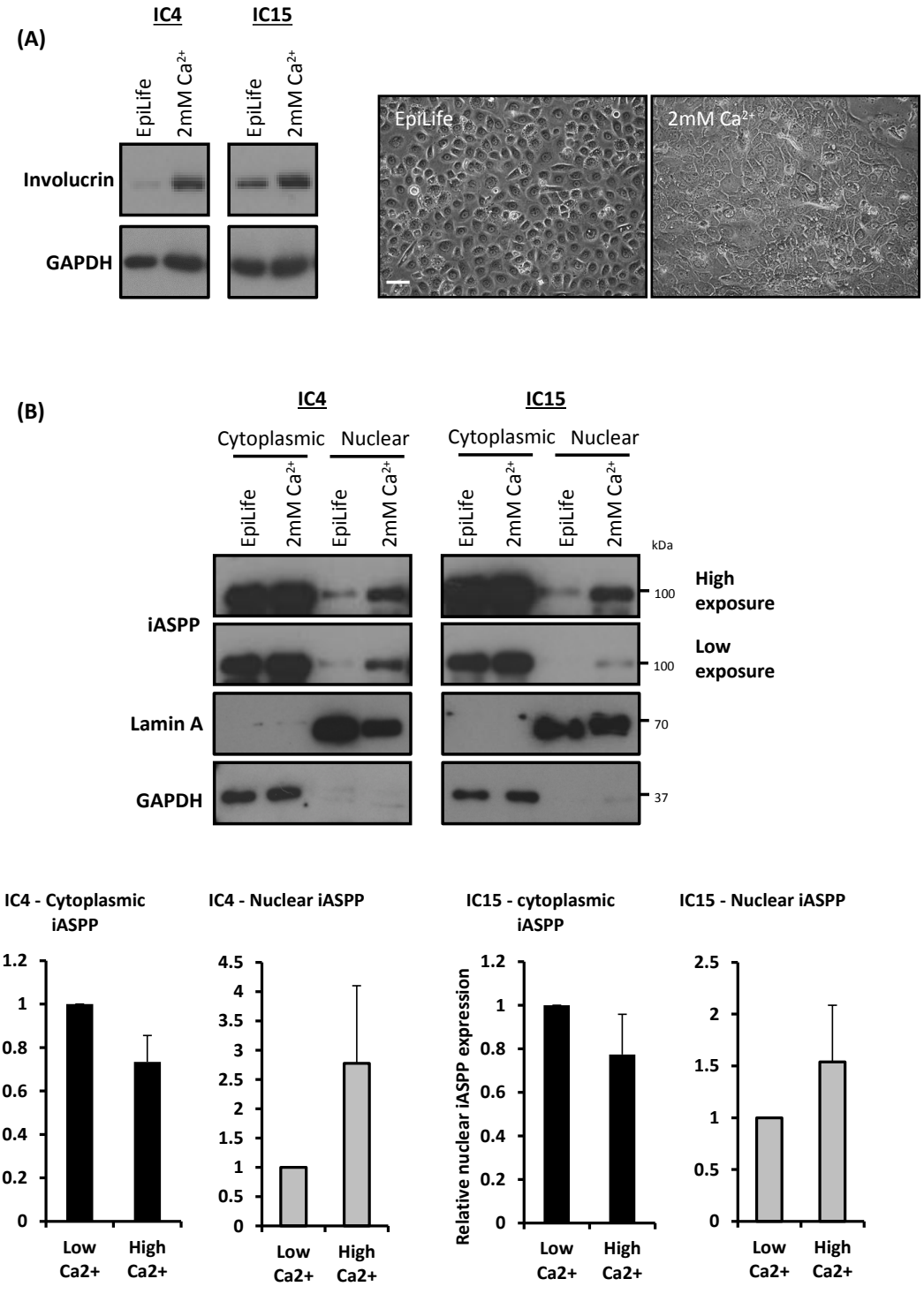


Figure 3.12. Calcium differentiated cSCC cells express higher levels of nuclear iASPP. (A) Involucrin levels of cSCC cell lines treated with 2uM Ca^{2+} for 48h. Representative images show cell line differentiation. Scale bars represent 20µm. **(B)** Subcellular fractionation experiments nuclear and cytoplasmic iASPP expression during low Ca^{2+} conditions (EpiLife) versus high Ca^{2+} conditions. High exposure and low exposure relate to the exposure of the film. Lamin A and GAPDH were used as controls for nuclear and cytoplasmic proteins respectively. **(C)** Densitometry analysis showing the

average iASPP protein expression levels when normalised to GAPDH or Lamin A. Error bars represent the SEM for three independent experiments.

3.3. Summary

Non-melanoma skin cancers (NMSC) are a huge and increasing health burden globally with substantial morbidity, mortality and costs to healthcare providers (Guy et al., 2015). cSCC is the second most common NMSC with more than one million cases a year in the US (Rogers et al., 2015). Thus, cSCC morbidity and mortality are largely unacknowledged and underestimated. Demonstrating that both iASPP and p63 are highly expressed in cSCC provides novel data on the molecular basis of squamous carcinogenesis.

The role of iASPP and p63 in cSCC was investigated using a panel of 10 cell lines generated from a varied population of patients with differing immunocompetence status, ages, genders and p53 and Notch1 mutations. An immortalised normal keratinocyte cell line (N-TERT) was used as a 'normal' control. p63 is found in several distinct isoforms. Consistent with published data, the predominant p63 isoform expressed in the cSCC cell lines was the isoform predominantly expressed in cancers due to its oncogenic tendencies, ΔNp63α (Yang et al., 1998; Parsa et al., 1999; Su et al., 2013). ΔNp63 was expressed highly at the protein and mRNA level in all the cSCC cell lines compared to N-TERT. The levels of ΔNp63 protein and RNA expression, however, did not appear to correlate with any of the features of the cell lines, including the differentiation status of the cell line.

To support this finding that ΔNp63 is highly expressed in cSCC cell lines I stained and scored 107 cSCC tumours for p63. As p63 is often highly expressed in the normal epidermis it was difficult to say whether there was an additional increase of expression in the tumours. Interestingly, a couple of previous reports highlighted that p63 expression may correlate with poorly differentiated SCC (Kargi et al., 2007; Alomari et al., 2014). In this chapter, however, all cSCC tumours showed a high percentage of cells stained for nuclear p63, regardless of tumour versus non-tumour cells or tumour differentiation stage. The data from this chapter therefore do not support previous suggestions that p63 expression may be used as a marker of poorly differentiated SCC (Kargi et al., 2007; Alomari et al., 2014). As Kargi et al. (2007) were working on poorly differentiated lung carcinomas in biopsy tissues and Alomari et al. (2014) stained only poorly differentiated cSCC tumours, their observations about p63 and the differentiation status of the cell may differ. My findings support previous discoveries that strong nuclear p63 staining is present in cSCC (Reis-Filho et al., 2002; Wrone et al., 2004;

Dotto and Glusac, 2006). These data were further supported by immunofluoresence staining of p63 in the cSCC cell lines. In all cSCC cell lines p63 expression was strong and nuclear.

In parallel to p63 staining, iASPP expression patterns were also analysed in cSCC. Consistent with p63 expression data, iASPP expression at both the protein and RNA level was also high compared to N-TERT in all cSCC cell lines. The expression, however, could not be correlated with the mutational status of p53, Notch 1 or RAS, or differentiation status of the cell lines. In order to translate these findings into an *in vivo* setting, 106 cSCC tumours were stained and scored for iASPP protein expression. In the tumours I observed both high cytoplasmic/nuclear iASPP expression.

However, it was noted that the location of iASPP in the cell appeared to differ between tumour sections. Overall, the tumours contained less nuclear iASPP staining compared to the perilesional epidermis. Further investigation into this phenomenon found a correlation between nuclear/cytoplasmic iASPP staining and differentiation status. The well differentiated tumours contained higher levels of nuclear iASPP and lower levels of cytoplasmic iASPP compared to the poorly differentiated tumours and vice versa the poorly differentiated tumours expressed higher levels of cytoplasmic iASPP and lower levels of nuclear iASPP compared to well differentiated tumours. Lu et al. (2013) argued that nuclear iASPP was the active form of iASPP in melanoma cells. Data from this chapter show cytoplasmic iASPP is predominately expressed in cSCC and also its highest levels correlate with the worst prognostic cases (poorly differentiated cSCC). However, the strong cytoplasmic and weak nuclear staining of iASPP observed in the tumour set was not completely surprising as various studies beforehand had noted the oncogenic effects of cytoplasmic iASPP (Chen et al., 2010; Lu et al., 2010; Zhang et al., 2011; Jiang et al., 2011; Liu et al., 2012; Cao et al., 2013).

The location of iASPP was further investigated in the panel of cSCC cell lines. Recent reports contradict the function of nuclear versus cytoplasmic iASPP. In this chapter cytoplasmic iASPP was predominantly expressed in the more aggressive cSCC tumours. In melanoma and prostate cancer it has recently been reported that phosphorylation of iASPP at S84/S113 by cyclin B1/CDK1 prevents iASPP residing in a homodimer structure and enables monomeric iASPP to shuttle to the nucleus, resulting in increased p53 binding and inhibition (Lu et al., 2013; Morris et al., 2014). I therefore wanted to determine whether phosphorylated iASPP

would also be detectable in cSCC cells. Following the same protocol from Lu et al. (2013) I was unable to detect a higher molecular weight second band in cSCC cell lines and N-TERT. I included a melanoma cell line expressing the phosphorylated nuclear iASPP band as a positive control. Our data is supported by Lu et al. (2013) who also showed HaCaT cells displayed little or no phosphorylated iASPP. This suggests cell type specificity for this iASPP post-translational modification form. Immunofluorescence cell line data supported the observation that iASPP appeared to be mainly cytoplasmic in the cSCC cell lines. Further investigation into the location of iASPP via subcellular fractionation supported these findings showing high cytoplasmic expression in the cell. Interestingly the nuclear fraction observed in cSCC cell lines was the same molecular weight as cytoplasmic iASPP thus failing to show any upper phosphorylated band in the nuclear fraction. This could be either the consequence of different buffer used or suggest once more a cell type-specificity of iASPP phosphorylation. It also hints to a potential expression of an un-phosphorylated nuclear iASPP at least in this cell lineage.

In vitro experiments were performed to mimic the *in vivo* tumour data. cSCC cells were pushed into terminal differentiation via treatment with calcium to assess the effects of differentiation upon iASPP localisation. Subcellular fractionation of the differentiated cSCC cells showed higher nuclear expression in high calcium conditions compared to low. That is, when cells are well differentiated nuclear iASPP is high. These data were slightly contradictory to the pattern of expression of iASPP in the normal skin. In the normal skin, iASPP has mainly nuclear basal expression but becomes largely cytoplasmic in the differentiated epithelial cells (Notari et al., 2011). On the other hand this data correlates with the *in vivo* tumour data that well differentiated tumours express higher nuclear iASPP than poorly differentiated tumours.

In the normal epidermis, both iASPP and p63 displayed a nuclear (and cytoplasmic in the case of iASPP) phenotype. In normal skin samples p63 and iASPP colocalise and function via an autoregulatory feedback loop. Immunofluoresence analysis of both iASPP and p63 staining in cSCC tissue and cSCC cell lines found a lack of colocalisation between iASPP and p63. This therefore raises the question of whether this difference in location of iASPP and p63 in cSCC may cause the autoregulatory feedback loop to be disrupted and will be investigated in chapter 5.

Chapter 4: iASPP/p63 autoregulatory feedback loop in cSCC

4.1. Introduction and aims

The ASPP family members; ASPP1, ASPP2 and iASPP, all contain a similar C-terminal domain consisting of a proline-rich region, ankyrin repeats and a SH3 domain (Trigiante and Lu, 2006). It is this domain which proves to be the most important for binding partners of both iASPP and the other ASPP proteins (Iwabuchi et al., 1994; Bergamaschi et al., 2003). p53 preferentially binds to this domain along with p65/Rel A and Bcl-2, among others (Gorina and Pavletich, 1996; Sullivan and Lu, 2007). Later studies demonstrated the ability of iASPP to interact with the additional members of the p53 family - p63 and p73 (Robinson et al., 2008; Chikh et al., 2011; Notari et al., 2011). ASPP1 and ASPP2 bind p63 initiating the transactivation of p63 on the promoters of proapoptotic genes including *Bax*, *PIG3* and *PUMA* (Bergamaschi et al., 2004). Expanding on this work, Robinson et al. (2008) used solid-phase binding assays to demonstrate that iASPP was also able to bind p63 and surprisingly at a 3-fold higher affinity than ASPP2 binds p63. This led to the speculation that iASPP too was involved in the regulation of p63.

A recent report from our group has demonstrated a link between iASPP and p63 via an autoregulatory feedback loop encompassing two novel microRNA, miR-574-3p and miR-720, in the stratified epithelia (Figure 4.1) (Chikh et al., 2011). Briefly, it was found that, upon silencing of p63 in keratinocytes, the expression of iASPP was downregulated at both the protein and mRNA level. Conversely, when the expression of iASPP was inhibited, the protein expression of p63 was decreased but the mRNA levels were unaffected. Interestingly, it was discovered that this absence of change in p63 mRNA levels upon iASPP silencing was due to the expression of two microRNAs, miR-720 and miR-574-3p. MiR-574-3p and miR-720 levels increased when iASPP was silenced, in turn, causing a decrease in the expression of p63. These experiments provided evidence that miR-574-3p and miR-720 could inhibit p63 translation in keratinocyte cell lines. This feedback loop is essential for epithelial homeostasis and is implicated in various cellular processes including adhesion, differentiation and proliferation (Chikh et al., 2011).

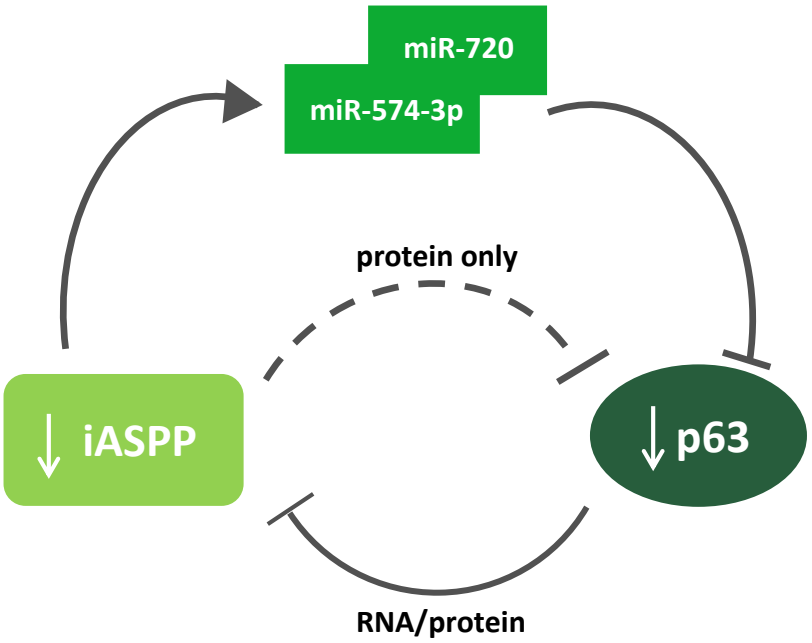


Figure 4.1. iASPP/p63 autoregulatory feedback loop in the stratified epithelium. Figure adapted from Chikh et al. (2011).

Further support for the iASPP/p63 axis came from iASPP knockout mice (Notari et al., 2011). iASPP was able to maintain cell homeostasis in the stratified epithelium of mouse skin and oesophagus via regulating the transcriptional activity of p63 (Notari et al., 2011). Additionally, studies on both NSCLC cell lines and mouse prostate epithelium showed the effect of iASPP on p63 (Cai et al., 2012b; Morris et al., 2014). In the prostate epithelium, iASPP knock out mice exhibit a decrease of p63-expressing basal cells. It is of note that in prostate cancer where loss of p63 is a well-documented event, nuclear iASPP expression increased (Morris et al., 2014). Further investigation into this phenomenon has yet to be carried out.

A handful of key reports have shown the importance of iASPP and p63 in the epithelium, in particular, the stratified epithelium of the skin (Notari et al., 2011; Chikh et al., 2011; Morris et al., 2014). Taking these data into account it was deemed important to investigate the interaction of iASPP and p63 in a system where the normal homeostasis of the skin is dysregulated. In this project cSCC has been used as a model. In the previous chapter, iASPP and p63 were highly expressed in both cSCC tissues and cSCC cell lines. Additionally, the location of iASPP differed in cSCC tissue compared to normal skin. For these reasons it was

hypothesised that the iASPP-p63 feedback loop previously characterised in human normal/immortalised keratinocytes may have become dysregulated in cSCC. During dysregulation, the levels of iASPP and p63 would no longer be controlled. Taking this hypothesis into consideration a dysregulated system in cSCC could provide a target/targets for designing much needed therapeutics combating cSCC. Alternatively a component of this hypothesised dysregulation could be used as a potential marker for cSCC.

Aims for this chapter:

1) Identify a suitable silencing technique for p63 and iASPP in cSCC cell lines
2) Establish whether iASPP and p63 are still able to affect the expression of each other via the feedback loop in cSCC cell lines and determine the effect of iASPP nuclear/cytoplasmic location upon this
3) Confirm whether the two microRNA identified in the initial feedback loop (miR-574-3p and miR-720) are still involved in p63 regulation via iASPP
4) Discover any additional microRNA in cSCC, in relation to iASPP and p63, using a microRNA array

4.2. Results

4.2.1. Identification of suitable silencing techniques for iASPP and p63 in cSCC cell lines

To facilitate the investigation into iASPP/p63 in cSCC cell lines, the levels of iASPP and p63 expression needed to be manipulated. Various techniques are available for the silencing of genes within cell lines. Chikh et al. (2011) used small interfering RNA (siRNA) against iASPP and p63 in HaCaT, N-TERT and normal human keratinocytes but were also able to stably transfect both HaCaT and N-TERT cells with short hairpin RNA (shRNA). ShRNA silencing enabled a more in-depth investigation of the feedback loop. Considering this, a robust silencing system to allow the investigation into iASPP and p63 in cSCC had to be established.

ShRNA are stem-loop RNA structures comprising of two complementary 19–22 base pairs connected by a loop of 4–11 nucleotides able to enter the cell via a viral vector (Moore et al., 2014). Once inside the cell shRNA is reverse transcribed and directly integrated within the DNA in the nucleus. Here, the shRNA is transcribed and exported out into the cytosol where it is processed by the enzyme, dicer, into siRNA duplexes. The siRNA binds to target mRNA which is subsequently degraded by the RNA induced silencing complex (RISC) (Moore et al., 2014). For the purpose of the current research, stably knocked down cells via shRNA were generated. Stably transfected cell lines have a high percentage of silenced cells and the silencing is sustained for many passages. In accordance with this, HaCaT, N-TERT and three individual cSCC cell lines; IC4, IC15 and IC18 were stably knocked down for iASPP using Dharmacon® SMARTvector® 2.0 Lentiviral shRNA particles. As this project is predominantly focussing on iASPP, only shRNA against iASPP was purchased. The Dharmacon® SMARTvector® 2.0 system provides ready-to-use viral particles eliminating the need to produce the lentivirus via packaging plasmids and a packaging cell line. Three unique shRNA constructs were tested against iASPP. Lentivirus was chosen over retrovirus (used by Chikh et al. 2011) as previous studies in our laboratory had found that stably knocking down iASPP in an alternative cancer cell line (melanoma) with retroviral particles resulted in the knockdown only lasting for 1-2 passages. Lentiviruses are a subtype of retrovirus, but unlike retrovirus, are able to infect both dividing and non-dividing cells, enabling a larger selection of cell types to be targeted (Song and Yang, 2010).

Only cells with a low passage number were used for the present experiment as the transduction efficiency of the lentivirus is greater in these conditions. The number of lentiviral transducing units to be added was optimised using a multiplicity of infection (MOI)

of 2. Cells passaged post-transduction were positively selected with puromycin due the presence of a puromycin resistance gene. In addition, passaged cells were actively transfected with the viral particle, that is, they were able to fluoresce green due to a GFP tag. Two out of the three unique shRNA constructs were able to produce at least a 50% knockdown in protein expression (Supplementary Figure 4.1 – Chapter 8, Appendix page 222). Despite these positive outcomes, the cell had seemingly developed a system to overcome the silencing of iASPP after 1-2 passages. Unfortunately, the same passaging issue that occurred in melanoma cells with retrovirus also occurred in cSCC cells with lentivirus.

iASPP is an essential gene required for the cell to proliferate. In this scenario, it is plausible that the cell (in particular a cancer cell) is unable to function without iASPP and will develop a mechanism to overcome this silencing. It is also plausible that the silencing could be lost due to methylation of the promoter driving shRNA expression. The promoter used in this vector was a cytomegalovirus (CMV) promoter. Published research has demonstrated the ability of the CMV promoter to undergo methylation-dependent silencing (He et al., 2005).

Due to the lack of stable knockdown and for the purpose of this research, cells had to be transiently transfected with siRNA. SiRNA is a double stranded RNA molecule of approximately 21-23 base pairs long. SiRNA differs from shRNA as it is transfected straight into the cytosol and incorporated directly into the RISC. SiRNA is not integrated into the DNA and because of this it is a transient transfection. A pool of three validated Silencer® siRNA sequences (Ambion, Life Technologies) – siRNA-1; siRNA-2 and siRNA-3, were used for silencing all p63 isoforms and a pool of 4 ON-TARGET plus SMARTpool (Thermo Scientific) were used to silence iASPP. During the initial phase of optimisation a non-treated control as well as a non-targeting siRNA control was used. A non-treated control was included initially to ensure that no significant effects were observed on protein or mRNA levels when treated with the non-targeting siRNA.

SiRNA can be transfected into the cell via many routes (Fire et al., 1998; Reynolds et al., 2004). For this project both si-iASPP and si-p63 were transfected into the cell via transfection reagent DharmaFECT 1, a lipid based formula with low toxicity and high efficiency for most cell lines. Knockdown efficiency was optimised using differing concentrations of siRNA, differing volumes of transfection reagent and differing incubation time points. SiGLO, a transfection indicator was used as an initial means to analyse the toxicity versus efficiency of

DharmaFECT 1. It was deemed that a concentration of 50nM for 72h for si-iASPP and a concentration of 50nM for 48h for si-p63 were optimal (Supplementary Figure 4.2 - Chapter 8, Appendix page 223). The iASPP and p63 siRNA pools had previously been used in our group for other cell lines. To ensure no adverse effects the pools were deconvoluted and each individual siRNA was transfected into the cSCC cells, protein was extracted and samples were run on a 10% SDS-PAGE gel and blotted for iASPP/ΔNp63α (Figure 4.2A, 4.3A).

All p63 siRNA three constructs generated an effective knockdown of ΔNp63; however the si-pool marginally provided the best knockdown (Figure 4.2A). Although Chikh et al. (2011) had shown the effect of iASPP on both TAp63 and ΔNp63, the levels of TAp63 are almost undetectable at the protein level thus ΔNp63 was the primary focus of this chapter. In all three cell lines a strong knockdown was achieved using the p63 si-pool with a silencing efficiency of over 70% observed at both mRNA and protein level, as a result si-pool was used to the rest of the project (Figure 4.2B & C). Densitometry analysis of western blots was used to quantify the silencing efficiency at the protein level. Si-iASPP-1 and si-iASPP-4 demonstrated a slight knockdown at the protein level, however, the biggest knockdown was observed with the si-iASPP pool (Figure 4.3A). In all three cSCC cell lines a knockdown efficiency between 70-80% for mRNA and 60-90% for protein levels was observed, as a result si-pool was used to the rest of the project (Figure 4.3B and C).

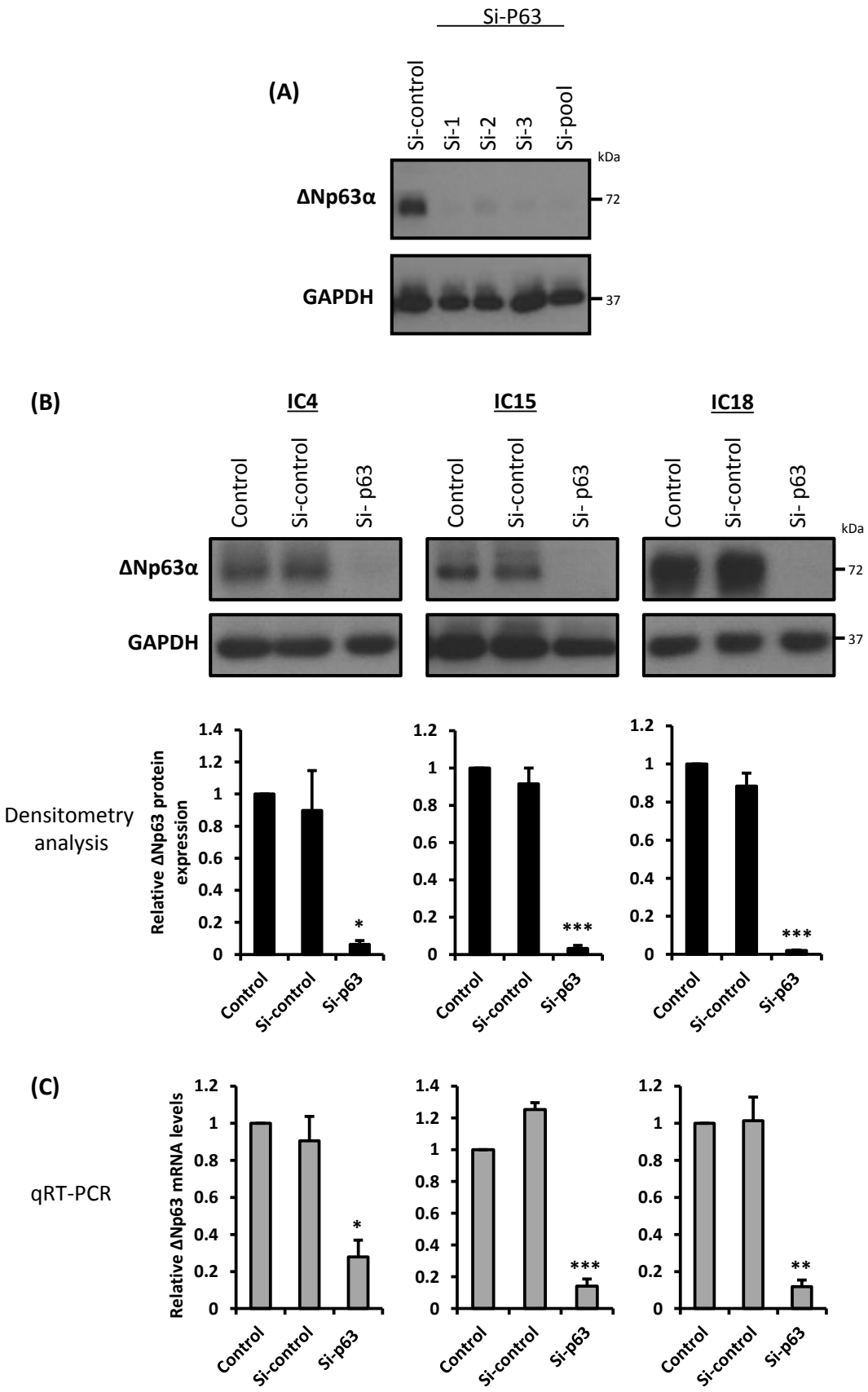

Si-P63
(A)
Si-control
Si-1
Si-2
Si-3
Si-pool
kDa
ΔNp63α
72
GAPDH
37
(B)
IC4
IC15
IC18
Control
Si-control
Si- p63
kDa
ΔNp63α
72
GAPDH
37
Densitometry analysis
Relative ΔNp63 protein expression
Control
Si-control
Si-p63
(C)
qRT-PCR
Relative ΔNp63 mRNA levels
Control
Si-control
Si-p63

Figure 4.2. Silencing of p63 in cSCC cells. (A) cSCC cells were either untreated (control), treated with a non-targeting control (si-control), silenced for p63 using individual siRNA (si-1, si-2, si-3) or si-pool. Protein was extracted and analysed by western blotting for p63 knockdown. GAPDH was used as a loading control. **(B)** Three cSCC cells (IC4, IC15, IC18) with control, si-control and si-p63 (si-pool) samples treated 50nM siRNA and lysed at 48h. Protein was analysed by western blotting for ΔNp63α. GAPDH was used as a loading control. Densitometry analysis shows the fold change compared to control in three independent experiments normalised to GAPDH. **(C)** QRT-PCR shows the fold change ΔNp63 mRNA levels of three independent experiments performed in triplicate relative to control. Si-control and si-p63 (si-pool) samples were treated with 50nM siRNA and lysed at 48h. Housekeeping gene GUS was used as an internal control. Error bars represent the SEM of three independent experiments. Statistical analysis was performed using a two-tailed, unpaired Student's *t*-test, comparing the si-control to si-pool. P-value < 0.05 = *, ≤ 0.01 = **, ≤ 0.001 = ***.

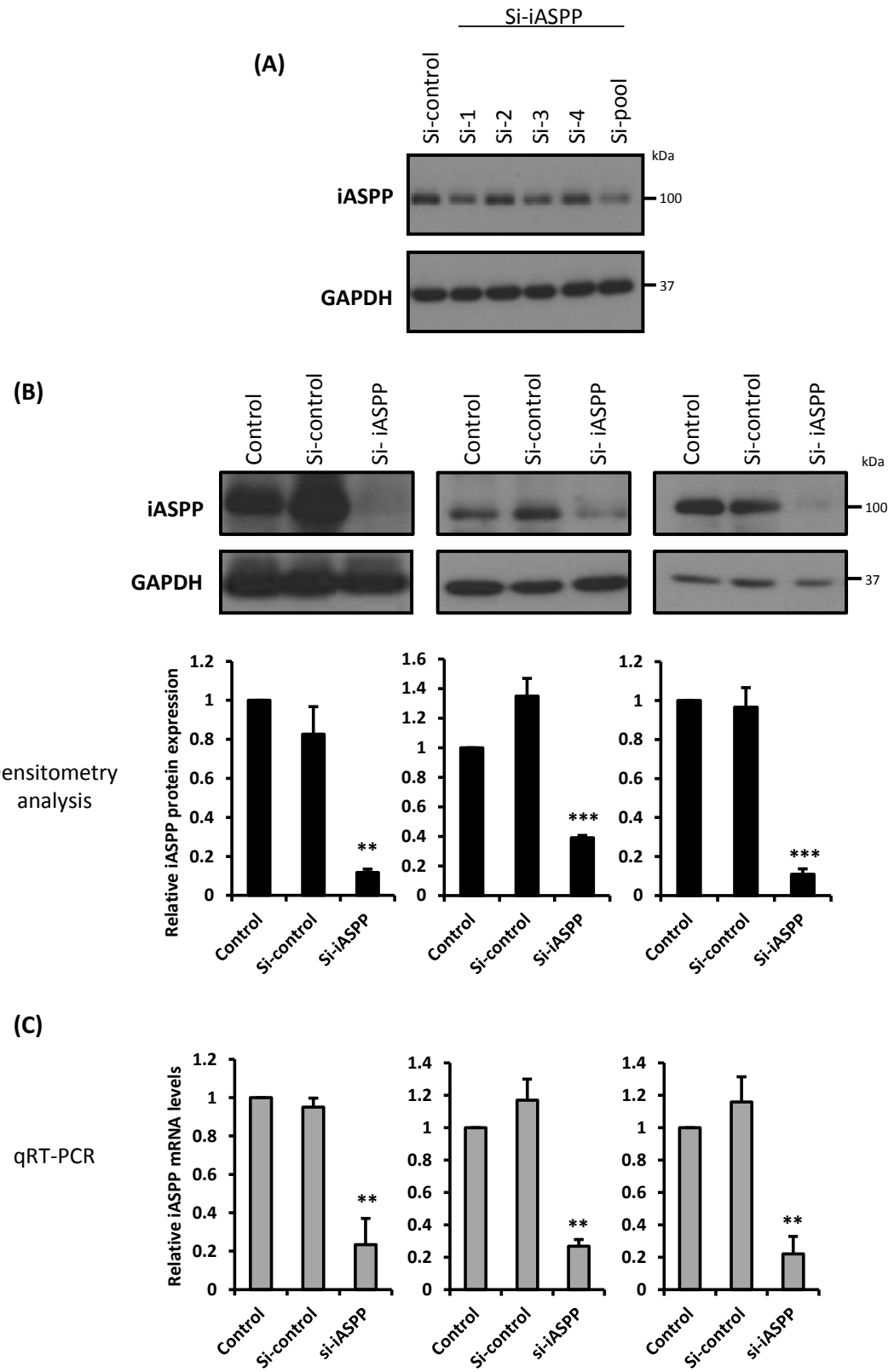

(A)
Si-iASPP
Si-control
Si-1
Si-2
Si-3
Si-4
Si-pool
kDa
iASPP
100
GAPDH
37
(B)
Control
Si-control
Si- iASPP
iASPP
GAPDH
kDa
100
37
Densitometry analysis
Relative iASPP protein expression
Control
Si-control
Si-iASPP
(C)
qRT-PCR
Relative iASPP mRNA levels
Control
Si-control
si-iASPP

Figure 4.3. Silencing of iASPP in cSCC cells. (A) cSCC cells were either untreated (control), treated with a non-targeting control (si-control), silenced for iASPP using individual siRNA (si-1, si-2, si-3, si-4) or si-pool. Protein was extracted and analysed by western blotting for iASPP knockdown. GAPDH was used as a loading control. **(B)** Three cSCC cells (IC4, IC15, IC18) with control, si-control and si-iASPP (si-pool) samples were treated with 50nM siRNA and lysed at 72h. Protein was analysed by western blotting for iASPP. GAPDH was a loading control. Densitometry analysis shows the fold change compared to control, in protein expression levels of iASPP in three independent experiments normalised to GAPDH. **(C)** QRT-PCR shows the fold change of iASPP mRNA levels of three independent experiments performed in triplicate normalised to control. Si-control and si-iASPP (si-pool) samples were treated with 50nM siRNA and lysed at 72h. Housekeeping gene GUS was used as an internal control. Error bars represent the SEM of three independent experiments. Statistical analysis was performed using a two-tailed, unpaired Student's *t*-test, comparing the si-control to si-pool. P-value < 0.05 = *, ≤ 0.01 = **, ≤ 0.001 = ***.

4.2.2. Validation of the iASPP/p63 autoregulatory feedback loop in N-TERT cells

Robinson et al. (2008) demonstrated the ability of p63 to bind to iASPP. This is supported by Chikh et al. (2011) who demonstrated, via chromatin immunoprecipitation, the ability of p63 to bind to the iASPP promoter in HaCaT cells. Despite conflicting evidence from Notari et al. (2011) and Cai et al. (2012b) who both failed to see an effect on p63 protein expression levels upon iASPP silencing, Chikh et al. (2011) found that reduction of p63 resulted in a decrease of iASPP at the protein and mRNA level. In addition reduction of iASPP led to a decrease in p63 at the protein level but not the RNA level (as it was later discovered that microRNA was controlling p63 translation).

This work was carried out in HaCaT cells and confirmed in N-TERT and primary human keratinocyte cells. To enable the investigation of iASPP and p63 into cSCC cells, validation experiments of previous observations carried out in our group were performed in N-TERT cells. Here, N-TERT cells were transiently silenced for iASPP and p63 expression, as discussed in the previous section, and the effects on ΔNp63 and iASPP protein and mRNA levels were evaluated via western blot and qRT-PCR (Figure 4.4A and B). In accordance with the earlier data, silencing of p63 in N-TERT cells confirmed reduction of the protein and mRNA levels of iASPP compared to si-control ($p=0.0011$). In addition, silencing of iASPP decreased the protein expression of ΔNp63α but not the mRNA levels. The previous research had demonstrated this lack effect of si-iASPP on ΔNp63 mRNA was due to microRNA control, this will be discussed in more detail in the next section.

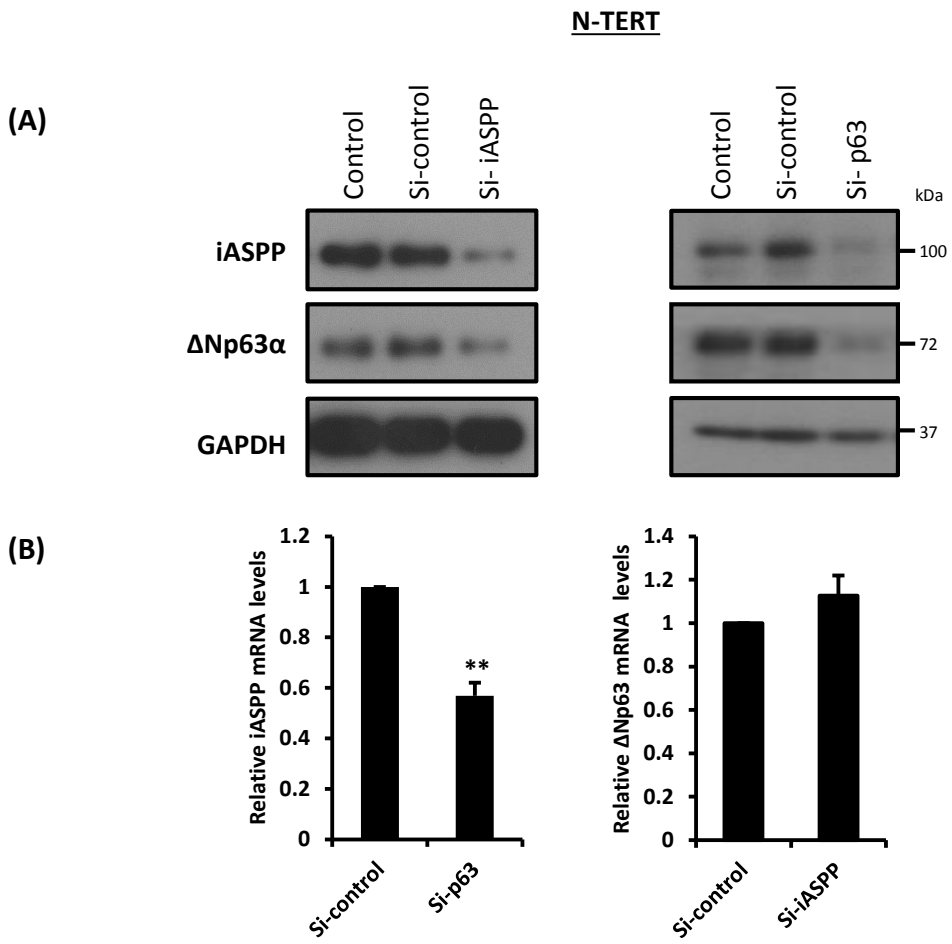


Figure 4.4. iASPP/p63 autoregulatory feedback loop in N-TERT cells. (A) N-TERT samples (control, si-control, si-iASPP/p63 - si-pool) were analysed for both iASPP/p63 protein expression by western blotting. GAPDH was used as a loading control. **(B)** mRNA from N-TERT si-control, si-iASPP/p63 (si-pool) samples analysed by qRT-PCR for iASPP/p63. Housekeeping gene GUS was used as an internal control. mRNA levels were normalised to si-control. Error bars represent the SEM of three independent experiments. Statistical analysis was performed using a two-tailed, unpaired Student's *t*-test, comparing the si-control to si-pool. P-value ≤ 0.01 = **.

4.2.3. In cSCC, p63 knockdown affects iASPP at the protein and mRNA level but iASPP knockdown affects p63 at only the protein level

The previous section had validated the published data and an appropriate system had been established to silence both iASPP and p63. The aim of this section was to discover if p63 and iASPP were functioning in the feedback loop in cSCC.

To investigate this work in cSCC, three cSCC cell lines were selected from a larger panel of well characterized human cSCC cell lines. Cell lines IC4, IC15 and IC18 were chosen for further study. The selection of these cell lines were based on several criteria. I wanted to select a sample set that would reflect the differences in cSCC in the general population to allow us to find a common mechanism enabling the discovery of a broad targeted therapy. All three cell lines were taken from individual patients (Table 4.1). IC4 and IC18 were derived from shin and the ear, respectively. As a result of the tumour site being a sun exposed region of the body both tumours are UV-induced. Interestingly, IC4 has wild type p53 and Notch1 and IC18 mutated p53 and Notch1. IC4 was derived from a well-differentiated tumour and IC18 from a moderately differentiated tumour.

Table 4.1 Characterisation of cSCC cell lines

Cell line	Gender	Age	Immune status	Tumour Site	Differentiation status of tumour	p53 mutation	Notch1 mutation
IC4	Female	73	Immunocompetent	Shin	Well	Wild type	Wild type
IC18	Male	81	Immunocompetent	Ear	Moderate	Mutation	Mutation
IC15	Male	73	Immunocompetent	Penis	Moderate	Wild type	Mutation

IC15 was chosen for further study as the initial tumour site for IC15 was on the penis. As the location of this tumour is not classically a sun exposed area (as with most cSCCs) the tumour was genotyped by PCR and was found to be an HPV-16 infected cSCC (Karin Purdie, unpublished data). The human papillomavirus (HPV) family contain several high-risk HPVs

known to cause cancer. Nearly all cervical cancer is caused by HPV with 70% being caused by high-risk HPV-16 and 18 and several other cancers are associated with HPV including anal, oropharyngeal, vulvar, vaginal and penile cancers ((Winer et al., 2006; Gillison et al., 2008). IC15 has wild type p53. HPV-16 infected tumours do not tend to have mutant p53 as E6-AP is able to cause wild type p53 proteasomal degradation (Scheffner et al., 1990; Huibregtse et al., 1991; Busby-Earle et al., 1994). Notch1 mutations display a similar pattern; in HNSCC wild type Notch tends to be present in a higher proportion of HPV-positive tumours (Rettig et al., 2015). In this case however IC15 cell line does have mutated Notch1.

Both IC4 and IC15 express reasonably high levels of both iASPP and p63. IC18 has lower levels of expression of both (Figure 3.2). ΔNp63 and iASPP protein and mRNA levels were analysed when iASPP and p63 were respectively silenced in cSCC (Figure 4.5 and 4.6). The data displayed similar results to those generated in section 4.2.2 from N-TERT cells and the previous research by Chikh et al. (2011). When p63 was silenced in cSCC cells this significantly reduced both the protein (IC4 $p=0.0219$, IC15 $p=0.0005$, IC18 $p=0.0001$) and mRNA levels (IC4 $p=0.00006$, IC15 $p=0.0022$, IC18 $p=0.0008$) of iASPP (Figure 4.5A and B). The analysis was performed by western blot and qRT-PCR respectively. This occurred in all of the three cell lines regardless of their mutation/UV status. Silencing of iASPP significantly reduced the protein levels of ΔNp63α (IC4 $p=0.0016$, IC15 $p=0.0012$, IC18 $p=0.0003$) but did not affect the mRNA levels of ΔNp63 (Figure 4.6A and B).

iASPP was originally identified as an inhibitor of p53 (Bergamaschi et al., 2001). p63 and p53 are able to bind to the same promotor region of iASPP, thus endogenous p53 expression was investigated in cSCC cell lines silenced for iASPP (Robinson et al., 2008). Protein lysate extracted from iASPP depleted cell lines was run on a 10% SDS-PAGE gel and blotted with a mouse monoclonal anti-p53 antibody (DO-1, Santa Cruz) able to react with both wild-type and mutant full length p53 (Figure 4.6C). In agreement with data previously published in normal skin (Chikh et al., 2011), there was no effect on full length p53 expression in all three cell lines regardless of the mutation state (Figure 4.6).

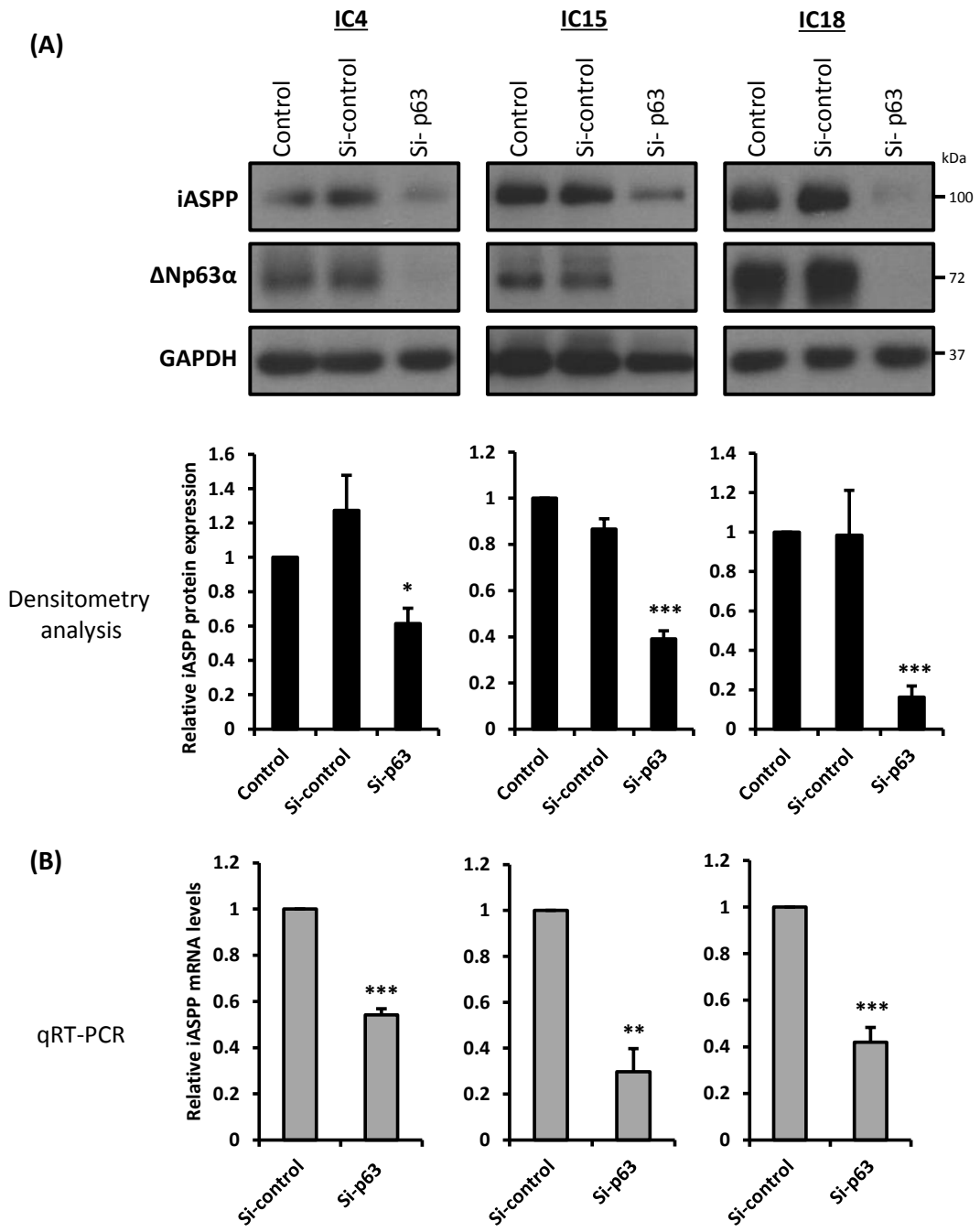


Figure 4.5. Silenced p63 reduces iASPP expression in cSCC. (A) Samples from three cSCC cell lines (control, si-control, si-p63 - si-pool) were analysed for both iASPP/ΔNp63α protein expression by western blotting. GAPDH was used as a loading control. Densitometry analysis shows the fold change, compared to control, in protein expression levels of iASPP in three independent experiments normalised to GAPDH. **(B)** mRNA from si-control, si-p63 (si-pool) samples analysed by qRT-PCR for iASPP. Housekeeping gene GUS was used as an internal control. mRNA levels were normalised to si-control. Error bars represent the SEM of three independent experiments. Statistical analysis was performed using a two-tailed, unpaired Student's *t*-test, comparing the si-control to si-pool. P-value < 0.05 = *, ≤ 0.01 = **, ≤ 0.001 = ***.

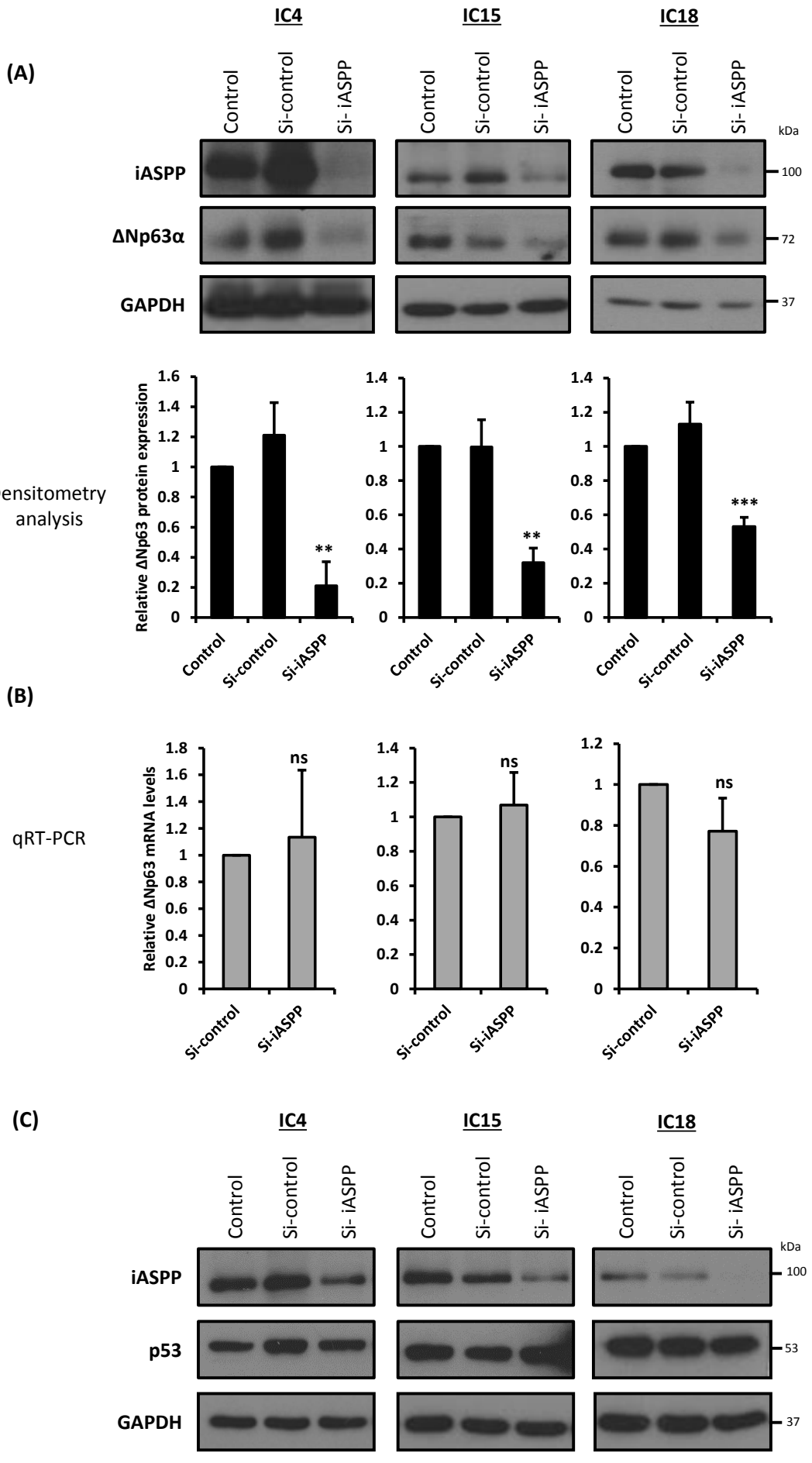

(A)
IC4
IC15
IC18
Control
Si-control
Si- iASPP
kDa
iASPP
ΔNp63α
GAPDH
100
72
37
Densitometry analysis
Relative ΔNp63 protein expression
**
**
***
Control
Si-control
Si-iASPP
(B)
qRT-PCR
Relative ΔNp63 mRNA levels
ns
ns
ns
Si-control
Si-iASPP
(C)
IC4
IC15
IC18
Control
Si-control
Si- iASPP
kDa
iASPP
p53
GAPDH
100
53
37

Figure 4.6. Silenced iASPP reduces ΔNp63α protein but not mRNA expression in cSCC, independently of p53. (A) Samples from three cSCC cell lines (control, si-control, si-iASPP - si-pool) were analysed for both iASPP/ΔNp63α protein expression by western blotting. GAPDH was used as a loading control. Densitometry analysis shows the fold change, compared to control, in protein expression levels of ΔNp63α in three independent experiments in three cSCC cell lines normalised to GAPDH **(B)** mRNA from si-control, si-iASPP (si-pool) samples analysed by qRT-PCR for ΔNp63. Housekeeping gene GUS was used as an internal control. mRNA levels were normalised to si-control. **(C)** Samples from three cSCC cell lines (control, si-control, si-iASPP - si-pool) were analysed for both iASPP/full length p53 protein expression by western blotting. GAPDH was used as a loading control. Error bars represent the SEM of three independent experiments. Statistical analysis was performed using a two-tailed, unpaired Student's *t*-test, comparing the si-control to si-pool. P-value ≤ 0.01 = **, ≤ 0.001 = ***.

4.2.4. Silenced p63 affects the expression of both nuclear and cytoplasmic iASPP

Reflecting on data in the previous section, it seems apparent that in our established cSCC cell lines the iASPP/p63 feedback loop is still intact. This is unexpected due to the fact that, as discussed in chapter 3, there appeared to be a difference between the location of iASPP and p63 in cSCC compared to the normal skin. In the tumour cells p63 is nuclear and iASPP predominantly cytoplasmic. To understand further the interaction of iASPP and p63 when iASPP is mainly cytoplasmic and p63 nuclear, a subcellular fractionation was performed in cSCC cells silenced for p63 (Figure 4.7). Previous research revealed that the C-terminus of iASPP, located in the nucleus, is responsible for binding members of the p53 family. The N-terminus of iASPP is responsible for its cytoplasmic location. When lacking the N-terminus, iASPP is located in the nucleus. In accordance with this, it was hypothesised that depletion of p63 would largely affect the nuclear form of iASPP (Slee et al., 2004; Robinson et al., 2009). This hypothesis, however, does not provide an explanation as to why the feedback loop still appears to be completely intact when iASPP is not always nuclear.

Results show that p63 is able to affect the expression of iASPP regardless of whether it is nuclear or cytoplasmic in three cSCC cell lines (Figure 4.7). When p63 is silenced both nuclear and cytoplasmic iASPP are decreased compared to the non-targeting control, although this is not statistically significant. Expression levels are analysed by western blot using lamin A and GAPDH as nuclear and cytoplasmic markers respectively. It is plausible that iASPP is affected by p63 in the nucleus and then shuttled out to the cytoplasm. However, further work would be required to investigate this.

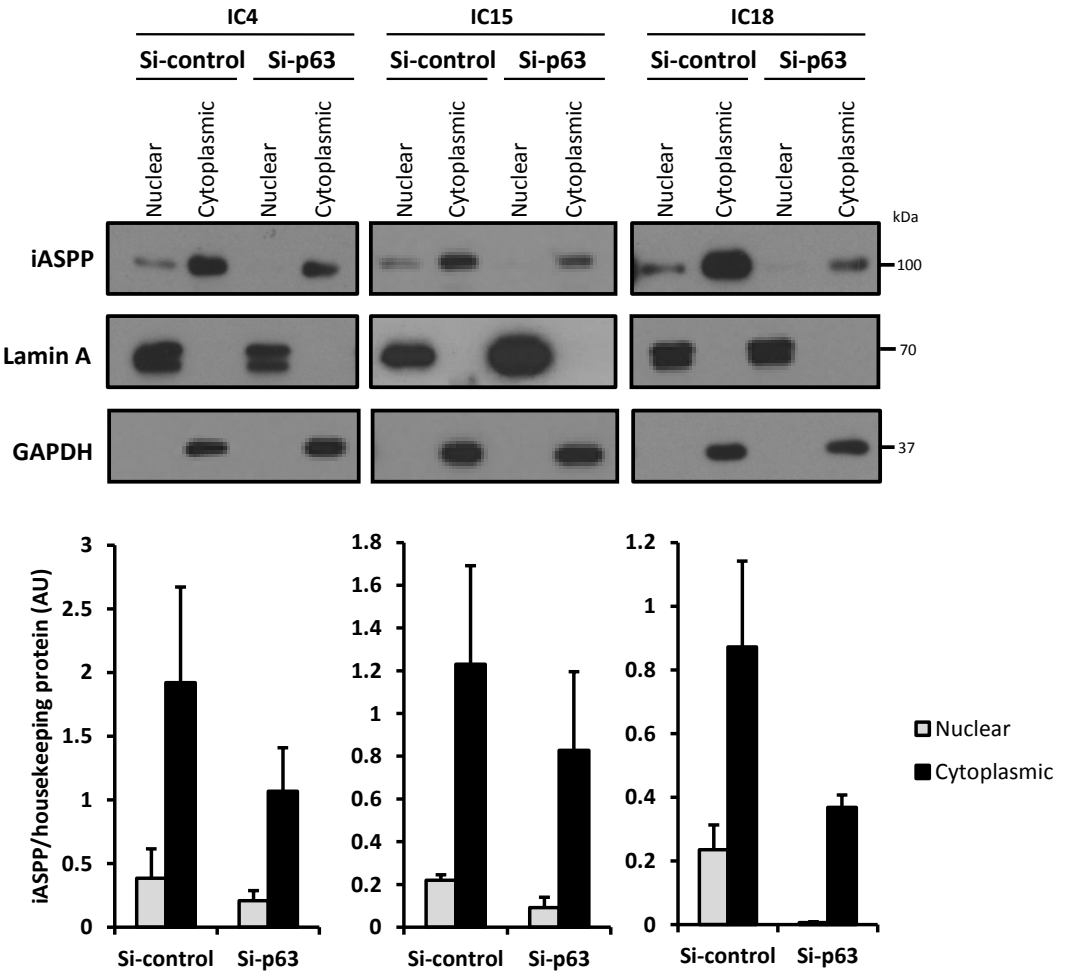


Figure 4.7. Silenced p63 reduces both nuclear and cytoplasmic iASPP. Cytoplasmic (C) and nuclear (N) proteins were extracted from cSCC cells untreated, or treated with si-control or si-p63 (si-pool). Protein lysate was run on a 10% SDS-PAGE gel and blotted for iASPP. Lamin A and GAPDH were used as controls for nuclear and cytoplasmic proteins respectively. Graphs show densitometry analysis of three separate experiments. Statistical analysis was performed using a two-tailed, unpaired Student's *t*-test, comparing the si-control to si-pool. Results were not statistically significant.

4.2.5. Silencing of iASPP does not appear to increase miR-574-3p or miR-720 in cSCC

Due to the high expression levels of iASPP and p63 in cSCC it was surprising to us that the evidence obtained at this stage showed the iASPP/p63 feedback loop was not dysregulated. However, further investigation into these findings was required. Consistent with the previous findings reported, upon silencing iASPP, p63 mRNA expression was not affected but p63 protein levels were significantly reduced. To rule out degradation of p63 protein, cells were treated with MG132, an inhibitor of proteasome-mediated degradation. This treatment did not restore expression of p63. Chikh et al., (2011) had described that silencing of iASPP in HaCaT cells increased endogenous expression of two microRNA, miR-574-3p and miR-720, which in turn, were essential for controlling p63. The combination of high levels of iASPP expression and the observation of low microRNA levels in the cSCC tumours would complement this finding.

Prior to assessing the role of miR-574-3p and miR-720 in cSCC, silencing of iASPP was performed in N-TERT cells to observe an increased level of microRNA as previously reported (Figure 4.8A). This result however, has not been replicated. In this project no increase in miR-574-3p or miR-720 is observed in N-TERT cells after iASPP depletion (Figure 4.8A). As in the previous study, microRNA levels were analysed via TaqMan qRT-PCR assays. RNU48, part of a small-nucleolar RNA (snoRNA) group of small noncoding RNAs, was used to normalise C_T values generated by microRNA, as RNU48 had high C_T values across different tissue types and displayed a low variability (Applied Biosystems; Mattick and Makunin, 2005)

As the published findings by our group on the microRNA were performed in the HaCaT cell line, the experiment was repeated in HaCaT but with still no increase in microRNA after iASPP silencing (Figure 4.8B). A plausible reason to explain the difficulty in reproducing this finding could be that the previous data were performed with HaCaT cell lines stably knocked down for iASPP. Given that the target sequences of shRNA and siRNA are different could help to explain this anomaly. Perhaps unsurprisingly when the same experiments were performed in the cSCC cell lines there was also no increase observed in the microRNA levels when iASPP was silenced. The only slight increase observed was in miR-720 in IC18 cells; however this was not significant (Figure 4.8B). Although in the case of cSCC the overall lack of effect could potentially be explained by a loss of regulation by iASPP on the microRNA, these claims cannot be confirmed whilst the initial results in HaCaT cells are not reproducible.

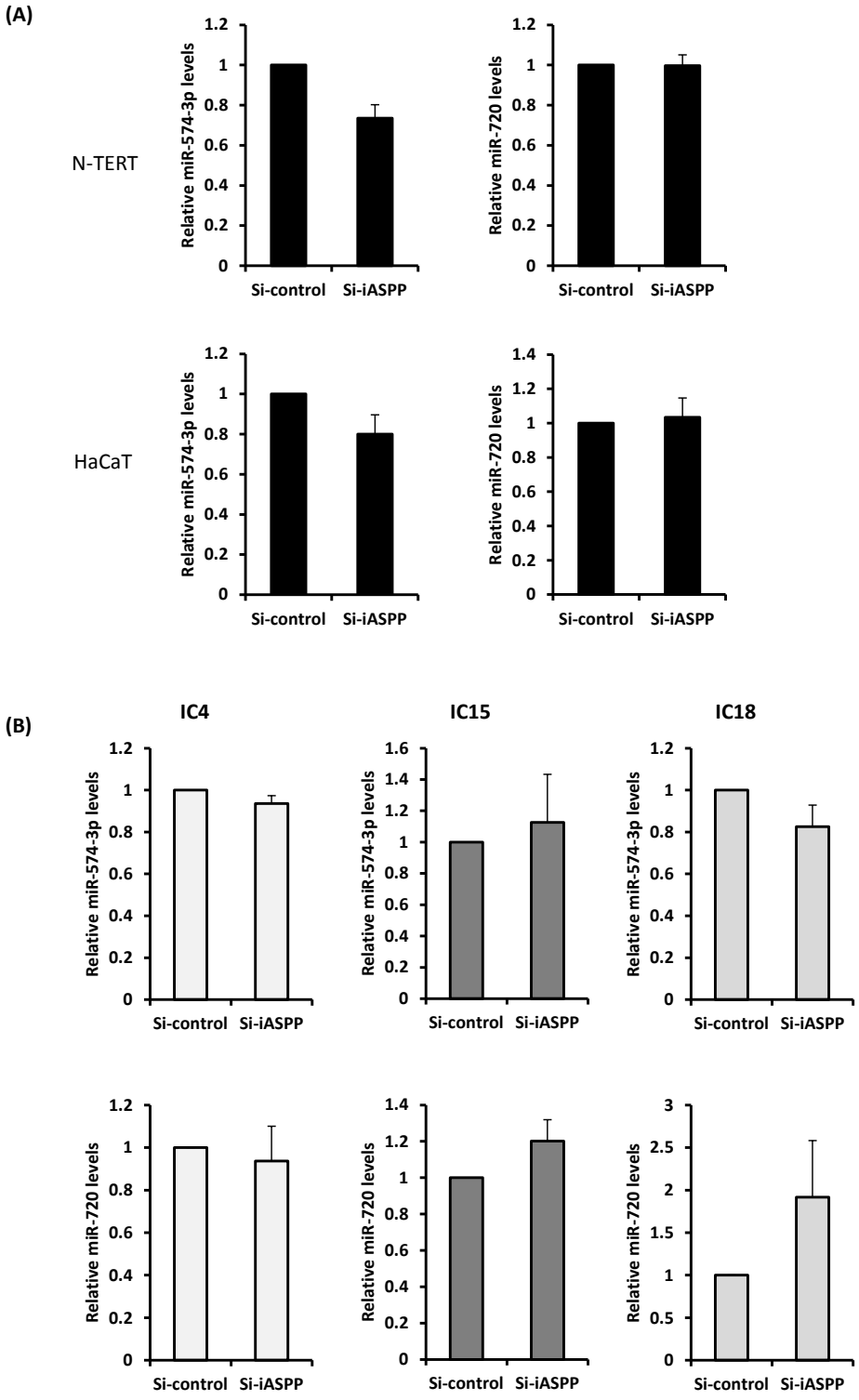


Figure 4.8. Effect of silencing iASPP on microRNA levels in N-TERT and HaCaT versus cSCC cell lines. **(A)** N-TERT and HaCaT cells either treated with si-control or si-iASPP (si-pool) screened for miR-720 and miR-574-3p levels by qRT-PCR. **(B)** Three cSCC cell lines (IC4, IC15, IC18) either treated with si-control or si-iASPP screened for miR-720 and miR-574-3p levels by qRT-PCR. RNU48 was used as an internal control. mRNA levels were normalised to si-control. Error bars represent the SEM for three independent experiments.

4.2.6. In situ *hybridisation staining of miR-574-3p and miR-720 shows low level expression in cSCC tissues*

As an alternative way to investigate the effect of iASPP on miR-574-3p and miR-720, I collaborated with colleagues from the University of Cologne, who had worked in the previous paper by Chikh et al. (2011). Previously our collaborators had shown a cytoplasmic and suprabasal expression of miR-574-3p and miR-720. Here, *In situ* hybridisation was performed in a series of cSCC human tumours to detect the levels miR-574-3p and miR-720. *In situ* hybridisation enables the detection of microRNA in tissue. The same probes designed for the initial study from Exiqon were used. Locked nucleic acids used in the detection probes are highly specific and have a higher sensitivity compared to standard DNA probes (Válóczi et al., 2004).

Eight cSCC tumours (three well, three moderate and two poorly differentiated) were compared to three sections of 'normal' skin (taken from breast reductions and an abdominoplasty). The staining of the sections were analysed by myself and Dr Martin Hufbauer, from the University of Cologne, for the intensity of cytoplasmic staining and the presence of nuclear staining. Of note is the observation that compared to the original staining in Chikh et al. (2011) additional nuclear staining in the normal skin can be reported (Figure 4.9). The protocol had since been optimised and refined providing a possible explanation for this difference. Unfortunately there was also a technical issue relating to the counterstaining of the sections with haematoxylin, another explanation as to why the normal skin staining differs from that published by Chikh et al. (2011). Regardless of these differences, the cSCC tissue staining showed that, irrespective of differentiation status, all tumours had a weak cytoplasmic staining of miR-574-3p and miR-720 and thus a decrease in the microRNA levels compared to the normal skin controls (Figure 4.9). The presence for high levels of iASPP and p63 in the cSCC tumour and a decrease of microRNA levels could perhaps signify that iASPP is controlling the microRNA. However this was a small sample size.

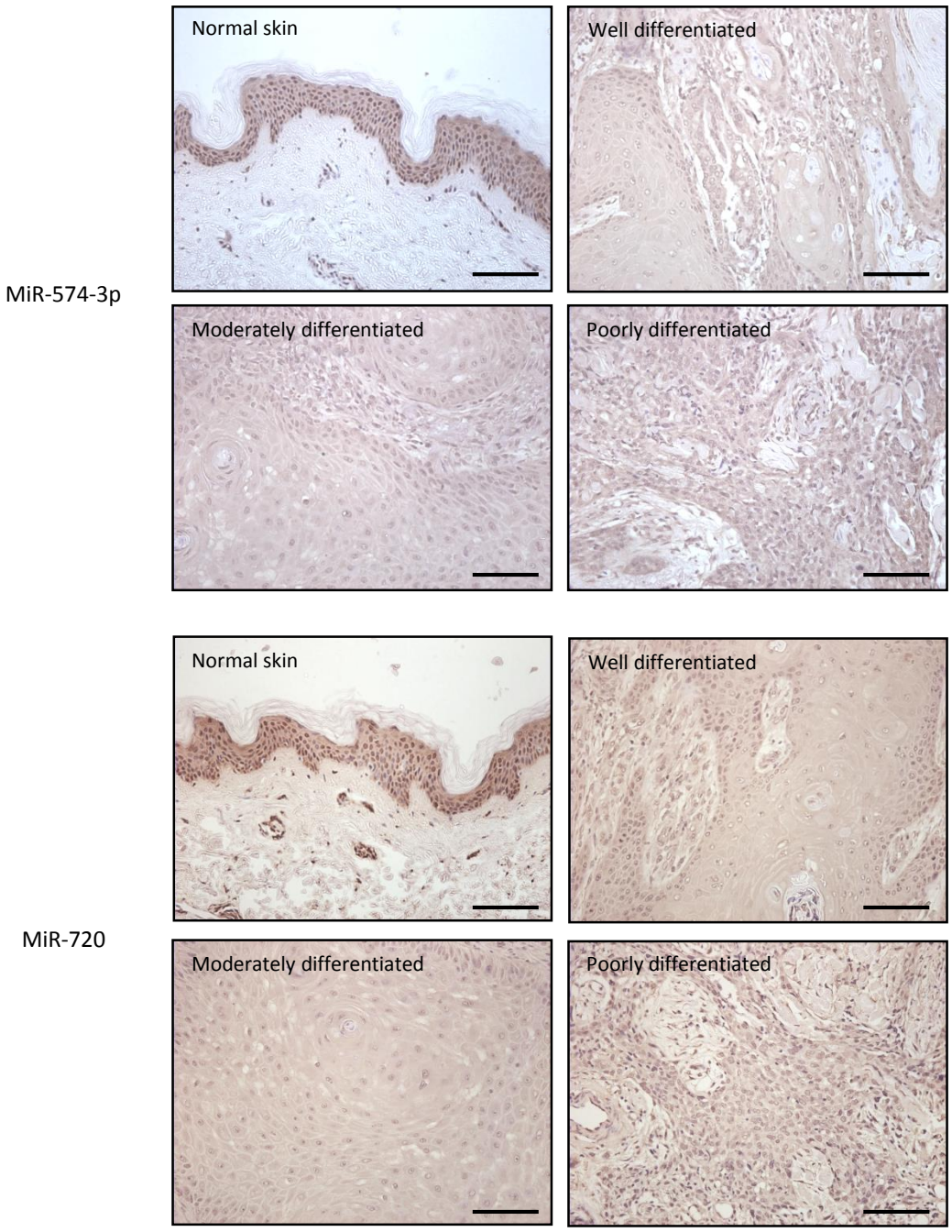


Figure 4.9. *In situ* hybridisation staining of miR-574-3p and miR-720 shows low level expression in cSCC tissues. (A) Representative images of *in situ* hybridisation experiment performed on normal human skin and human cSCC sections. Dark brown staining represents positive staining for miR-574-3p and miR-720. Scale bar represents 100µM.

4.2.7. MiR-574-3p and miR-720 expression levels in cSCC cell lines

In a bid to investigate the role of miR-574-3p and miR-720 further, a panel of 10 cSCC cell lines and N-TERT were screened for miR-574-3p and miR720 expression. It was proposed that the screening of cSCC cell lines compared to N-TERT may provide a validation of the *in situ* data. MicroRNA levels were analysed via TaqMan qRT-PCR assays. RNU48 was used to normalise C_T values generated by microRNA. The screen showed an overall decrease in miR-574-3p levels in cSCC compared to N-TERT (Figure 4.10). Six out of the ten cell lines were significantly decreased (T1 p=0.00001, IC1 p=0.00002, T11 p=0.001, T8 p=0.00002). The exception is Met 1, which showed an increased level compared to N-TERT, although the error bars are large. This decrease in miR-574-3p levels was expected due to the earlier finding in this report that p63 and iASPP are highly expressed in cSCC and is consistent with the *in situ* data. Higher iASPP expression is able to inhibit the microRNA activity and upregulate p63 expression indicating a loss of control of the microRNA over p63.

In contrast, an overall increase was observed in miR-720 levels in cSCC cell lines compared to N-TERT (Figure 4.10). However, this increase was not significant. These data are consistent with the qPCR data showing no relation of iASPP expression with miR-720.

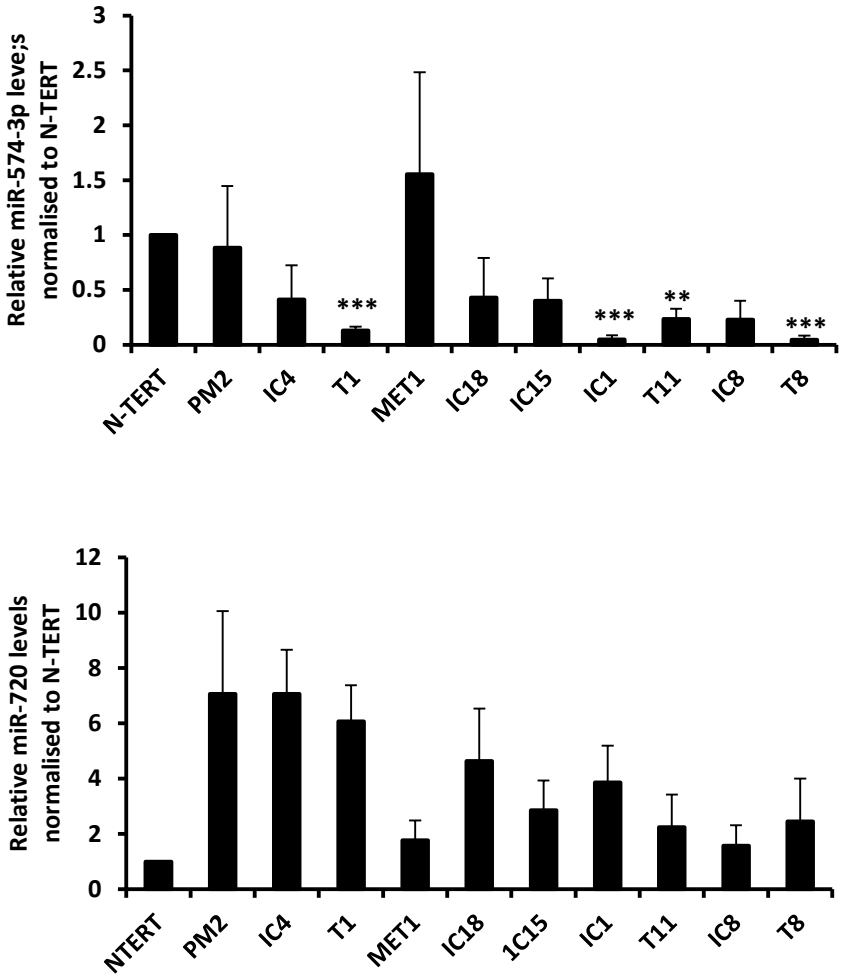


Figure 4.10. An overall decrease in miR-574-3p and an increase miR-720 levels are observed in a panel of cSCC cell lines compared to N-TERT. A panel of cSCC cell lines and N-TERT were screened for miR-574-3p and miR-720 levels by qRT-PCR. Graphs show the fold change relative to N-TERT. RNU48 was used as an internal control. Error bars represent the SEM of three independent experiments performed in triplicate. Statistical analysis was performed using a two-tailed, unpaired Student's *t*-test. Experiments have been corrected for multiple testing. P-value < 0.005 = *, ≤ 0.001 = **, ≤ 0.0001 = ***.

4.2.8. Pre-miR-574-3p and Pre-miR-720 treatment of cSCC cells decreased the expression of ΔNp63α

Data in the previous sections have been inconclusive. Silencing data had suggested iASPP was no longer controlling miR-574-3p and miR-720; however, this was unable to be validated. Conflicting data came from *in situ* hybridisation and cell line screen showing that cells with high levels of iASPP contained low miR-574-3p and miR-720, with the exception of the miR-720 cell line screen. Putting the interaction of iASPP with the microRNA aside, it was questioned whether the microRNA were still in control of p63. It was speculated that the high levels of p63 expressed in the tissue compared to the low levels of microRNA could signify a loss of microRNA control on p63.

To investigate this further three cSCC cells were transfected with Pre-miR-574-3p and Pre-miR-720. Pre-miR microRNA precursors, which act to mimic the effects of the microRNA, are taken up into the cell and processed via the RISC complex. Pre-miR microRNA precursors are transfected into the cell using DharmaFECT 1 in the same manner as siRNA transfection. Cells were transfected with 50nM Pre-miR microRNA Precursor and incubated for 48 hours. A Pre-miR-control was used as a negative control containing a random sequence unable to exhibit any effect on microRNA function. An untreated control was also included to ensure no unspecific effects of the Pre-miR-control. QPCR TaqMan assays were used to validate the effectiveness of transfection using RNU48 as an internal control (Figure 4.11A).

Western blot analysis of three cSCC cell lines transfected with Pre-miR-574-3p and Pre-miR-720 show an inhibitory effect on ΔNp63α protein levels in Pre-miR treated cells compared to controls (Figure 4.11B). Transfection with Pre-miR-574-3p showed a stronger reduction of ΔNp63α than Pre-miR-720 treatment. There was no effect on iASPP at the protein level when Pre-miR where transfected into the cell as the cells were only treated with Pre-miR for 48 hours – not enough time to see an effect on iASPP. These data confirm that, although in cancer cells it is unlikely that low levels of microRNA could still control high p63 levels, at least exogenous expression of miR-574-3p is still able to repress p63. Regardless of the interaction of iASPP with the microRNA these results could suggest a potential therapeutic route as a way of introducing microRNA control of p63 expression back into the tumour cell.

Experiments using anti-miR (microRNA inhibitors) were also undertaken to try to establish whether p63 was still being controlled by the microRNA in cSCC. Anti-miR blocks the activity

of the microRNA. Treatment with anti-miR in HaCaT cells by Chikh et al. (2011) managed to rescue p63 expression that had been reduced by silencing of iASPP. This experiment was tested in cSCC cell lines however, the same phenotype could not be observed and the anti-miRs looked like they may have had no effect (data not shown). It is noteworthy here though that trying to increase p63 levels in cSCC which were already very high would be difficult. Also, as these microRNA are expressed at low levels in cSCC, by blocking an already low microRNA level there potentially would not be much effect.

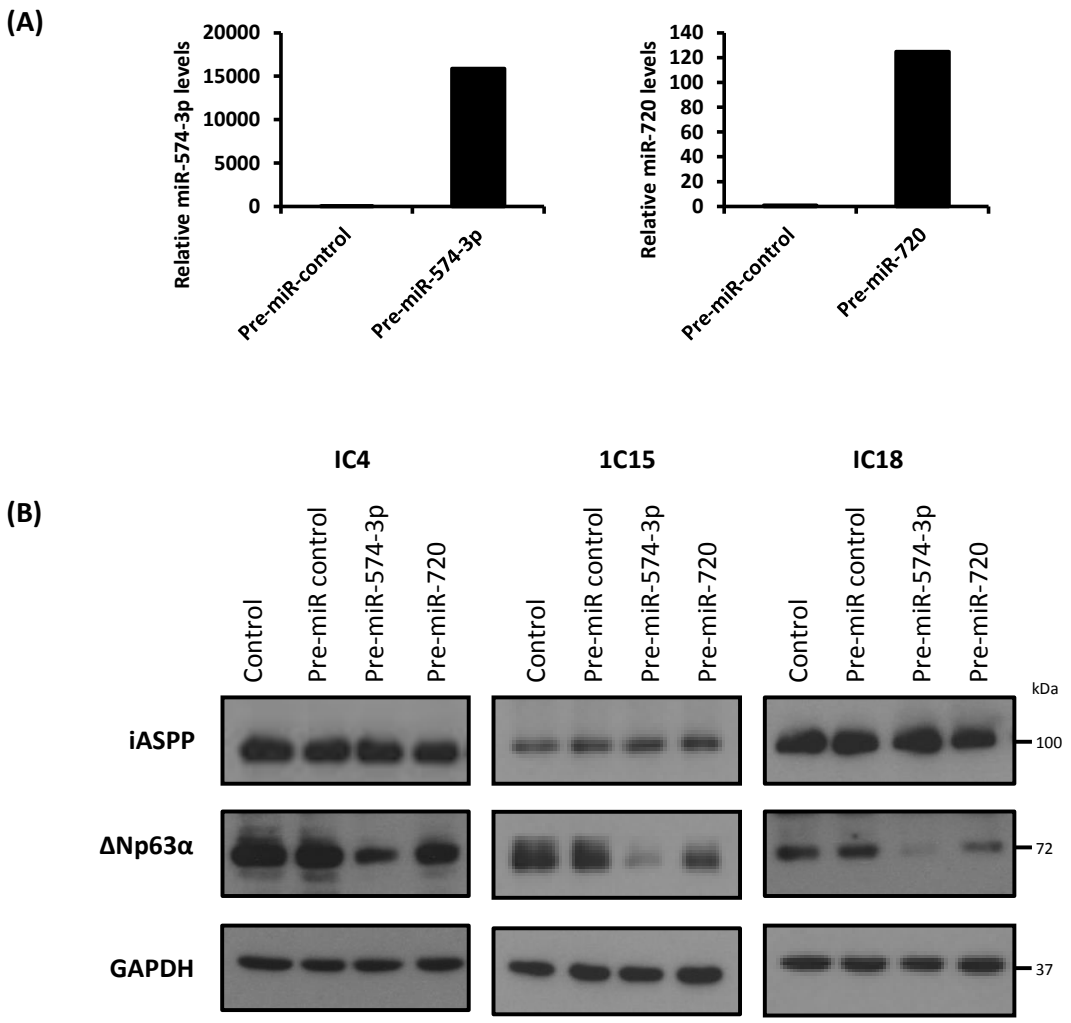


Figure 4.11. Treatment of cSCC cell lines with Pre-miR-574-3p/miR-720 decreases ΔNp63α expression. (A) QRT-PCR with IC4 cells was used to validate the effectiveness of Pre-miR-574-3p and Pre-miR-720 treatment. Graphs show the fold change Pre-miR treated cells compared to control. **(B)** Cells untreated (control), treated with a non-targeting control (Pre-miR control), treated with Pre-miR-574-3p or Pre-miR-720. Protein lysates were run on western blot and ΔNp63α protein expression was determined. GAPDH was used as a loading control**.**

4.2.9. MicroRNA array on cSCC cell lines

To clarify the results from previous sections regarding the effect of iASPP on microRNA in cSCC, a microRNA array was undertaken. For this experiment I cultured and transfected the cells, and extracted the microRNA. The microRNA array was then performed by Dr Giovanna Chiorino, Cancer Genomics Lab, Italy. RNA from three cSCC cell lines, IC4, IC15 and IC18 treated with either non-targeting siRNA or si-iASPP were analysed along with N-TERT cells treated with either non-targeting siRNA or si-iASPP. Samples were hybridised to oligonucleotide glass arrays with sequences representing probes for 2006 human microRNAs from the Sanger database v19 (Human microRNA 8x60K Microarray Version 19, Agilent Technologies). For each cSCC cell line and each unique probe, the non-targeting control signal was subtracted from the si-iASPP treated signal and a moderate *t*-test was applied to detect differentially expressed microRNAs. Slight modulation in terms of fold-change was accepted (+/- 1.25 FC) provided p-values were less than 0.01. A heatmap containing the normalised expression values of the significantly upregulated microRNA in IC4 and IC18 cell lines upon iASPP silencing is shown (Figure 4.12A).

During analysis of the microRNA array, it was noted that the cell line IC15 appeared to have a differing microRNA expression profile to the other two cSCC cells. When analysed together, 11 microRNA were significantly upregulated upon iASPP silencing in IC4 and IC18 (Figure 4.12A). When the fold changes of the same microRNA in IC15 were placed next to this data it was clear that these microRNA are not affected as in the other cell lines. When all three cell lines are analysed together only two microRNA are upregulated upon iASPP silencing. The reason for this difference could be due to the lineage of the IC15 cell line as the initial tumour site for IC15 was on the penis and is an HPV-16 infected cSCC. Due to the different origin of the IC15 cSCC cell line and thus the different genetic profile of this tumour, the focus for this project was on the IC4 and IC18 cell lines. The 11 upregulated microRNA identified in IC4 and IC15 were also compared to N-TERT. In the N-TERT cell line none of the microRNAs were upregulated suggesting a cSCC specific phenomenon.

The purpose of undertaking the microRNA array was to discover how iASPP was able to regulate p63 at the protein but not mRNA level. As Chikh et al. (2011) had found in normal skin this was via miR-574-3p and miR-720 it was proposed that these microRNA would be upregulated in the new array. However, here it is important to note that whilst performing this research it was noticed that the Sanger microRNA database 84 had updated to show that

miR-720 is no longer considered to be a microRNA but in fact a tRNA (Schopman et al., 2010). Schopman et al. (2010) published a report highlighting evidence suggesting that next generation sequencing approaches are susceptible to misannotations. In the case of miR-720, both miR-720 and $tRNA^{Leu}$ have an identical 18 nucleotide sequence leading to this tRNA-microRNA mimicry. More recent findings have described miR-720 as a 'microRNA-like' tRNA fragment or tRNA-derived microRNA where a mature tRNA is cleaved yielding small RNA fragments that have the ability to silence genes like microRNA (Haussecker et al., 2010; Guzman et al., 2015). Despite these updates, several reports relating to miR-720 have been published since with no reference to this finding. With regard to this body of work, although the miR-720 appears to still be able to act as microRNA when overexpressed in cSCC cell lines (Figure 4.11B), it cannot be considered in the new microRNA array. In addition to the above finding the miR-574-3p was not shown to be upregulated in the cSCC cells nor in N-TERT cells. These observations suggest that technical differences in silencing may affect results differently.

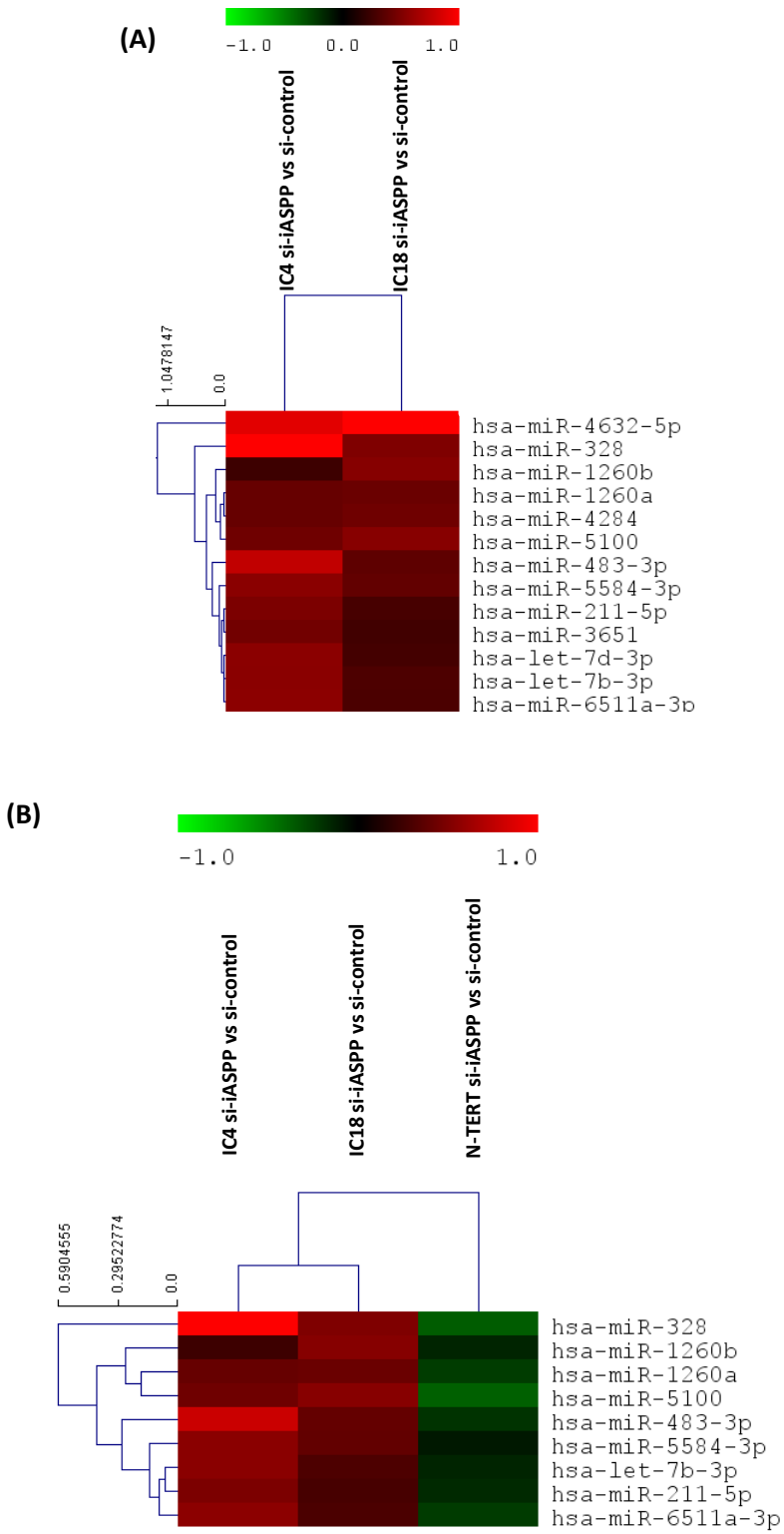


Figure 4.12. MicroRNA array cSCC cell lines. **(A)** Heatmap displaying Log-fold change values of microRNA detected in IC4 and IC18 cells. MicroRNA showing the greatest overexpression in cSCC cells treated with si-control versus si-iASPP (si-pool) are shown **(B)** Heatmap of microRNA showing the greatest overexpression in cSCC cells treated with si-control versus si-iASPP from IC4 and IC15, compared to N-TERT.

4.2.10. Identification and validation of microRNA targeting p63

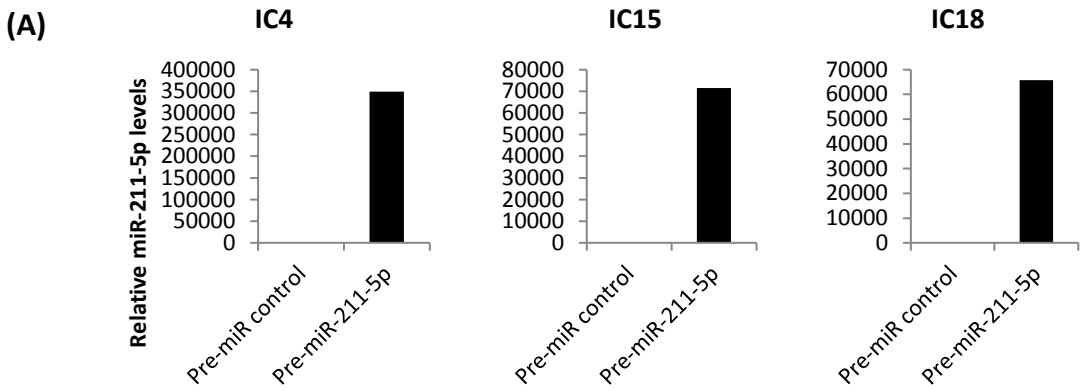
Before further validating the array findings by qRT-PCR it was important to determine which microRNA to focus on. From the 11 microRNA upregulated upon iASPP silencing, two microRNAs the miR-211-5p and the miR-328-3p were predicted to target p63 according to microRNA.org, miRDB.org and miRWalk 2.0. Additionally, a recent paper looking into microRNA feedback regulation of p63 in cancer progression speculated that miR-211-5p will target p63 (Lin et al., 2015).

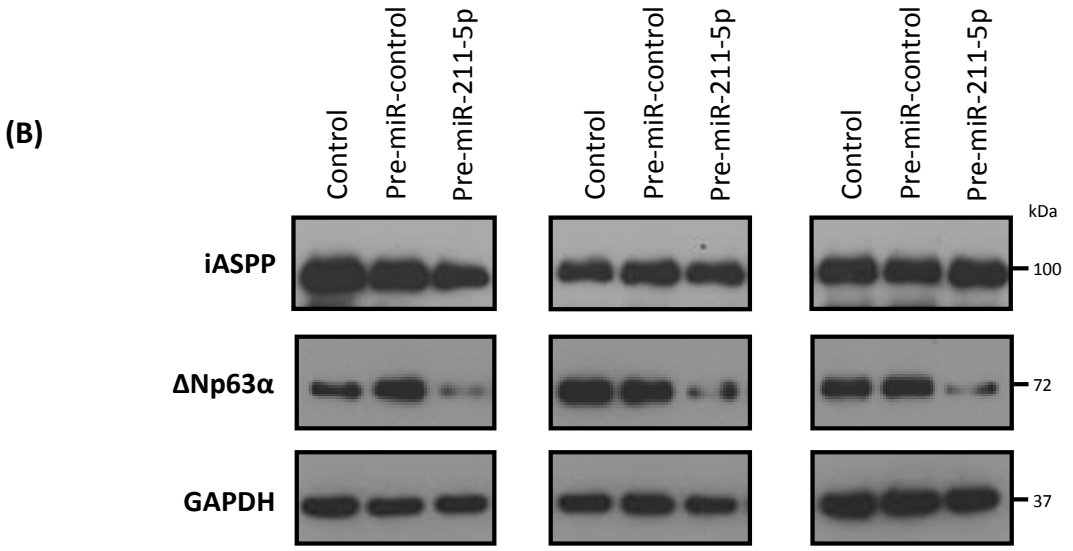
Data showing miR-211 behaving as an oncomir have been published for HNSCC, oral SCC and colorectal SCC (Chang et al., 2008; Cai et al., 2012a; Chu et al., 2013; Sümbül et al., 2015). Conflicting reports have come from melanoma, pancreatic and sebaceous carcinomas presenting miR-211 as a suppressor microRNA, suggesting a cell-type specific effect of miR-211 (Mazar et al., 2010; Levy et al., 2010; Sakurai et al., 2011; Boyle et al., 2011; Xu et al., 2012; Giovannetti et al., 2012; Maftouh et al., 2014; Tetzlaff et al., 2015). MiR-328 functions as a suppressor microRNA in glioblastoma, colorectal cancer, osteosarcoma breast cancer (Wu et al., 2012; Xu et al., 2012; Yang et al., 2014; Wang et al., 2015b; Yaun et al., 2015). An oncomir phenotype, on the other hand, has been associated with brain metastasis in NSCLC and glioma invasion (Arora et al., 2011; Delic et al., 2014). Additionally, conflicting reports in melanoma show miR-328 as both a suppressor and promoter in melanoma (Leidinger et al., 2010; Li et al., 2015). Until now (to the best of my knowledge), no report has linked miR-211-5p or miR-328-3p expression with a specific role in cSCC.

In order to test these predictions the cSCC cell lines were treated with Pre-miR against miR-211-5p and miR-328-3p and incubated for 48h to enable overexpression of miR-211-5p and miR-328-3p. To confirm overexpression, RNA was extracted and assayed by qRT-PCR (Figure 4.13A). Once the methodology had been confirmed, protein was extracted from the cells and run on a western blot using an antibody against p63, and GAPDH was used as a loading control (Figure 4.13B). In this case IC15 was included. Here, only treatment with the miR-211-5p was able to decrease the expression of p63. The effect of miR-211-5p was seen in all cell lines regardless of mutation/UV status. There was no effect on iASPP at the protein level when Pre-miR where transfected into the cell as the cells were only treated with Pre-miR for 48 hours – not enough time to see an effect on iASPP. The miR-328-3p had no effect on p63 (Supplementary Figure 4.3 – Chapter 8, Appendix page 224).

To date, no reports have been published showing miR-211-5p targeting p63. A 3'UTR luciferase assay was performed to determine whether the miR-211-5p was able to interact specifically with p63 (Figure 4.13C). A Luciferase assay is able to detect the effect of microRNA on a target gene. Here, the 3'UTR of p63 was cloned into a PSI-check 2 vector containing *Renilla* luciferase, *hRluc*, as a primary reporter gene. The *Renilla* luciferase reporter gene enables the detection of changes as a result of microRNA inhibition. When the microRNA binds to the 3'UTR of its target gene the mRNA is cleaved, decreasing the *Renilla* luciferase signal. If the microRNA is not specific for the 3'UTR of a gene the mRNA is translated and the *Renilla* luciferase signal emitted. A secondary firefly reporter expression cassette enables the normalisation of the *Renilla* (Jin et al., 2013; TECHNICAL BULLETIN siCHECK™ Vectors, Promega). To ensure specificity of the technique, a PSI-check 2 vector containing a mutant 3'UTR p63 was also developed and used as a negative control.

The end effect of the 3'UTR luciferase assay is a measure of *Renilla* luciferase signal. The 3'UTR luciferase assay was performed in HEK293 cells where there is little endogenous p63. In cSCC cells expressing high endogenous levels of p63, any transfected Pre-miR-211-5p into this system may become diluted by binding to endogenous p63 and not the 3'UTR in the vector, thus weakening the signal. The results show a 32% decrease in luciferase activity upon miR-211-5p overexpression with 3'UTR p63 ($p=0.00002$). The data confirm previous findings from this project that miR-211-5p is able to bind and target p63. This was further confirmed by the fact that a reduction in fluorescence was not observed in cells containing mutant p63 (Figure 4.13C).


(A)
IC4
IC15
IC18
Relative miR-211-5p levels
400000
350000
300000
250000
200000
150000
100000
50000
0
80000
70000
60000
50000
40000
30000
20000
10000
0
70000
60000
50000
40000
30000
20000
10000
0
Pre-miR control
Pre-miR-211-5p


(B)
Control
Pre-miR-control
Pre-miR-211-5p
kDa
iASPP
ΔNp63α
GAPDH
100
72
37

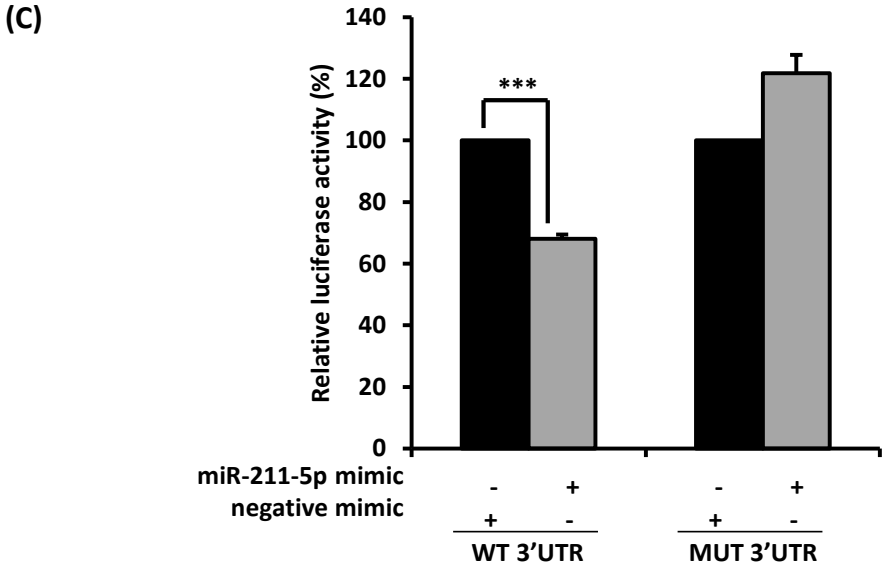

(C)
Relative luciferase activity (%)
140
120
100
80
60
40
20
0
***
miR-211-5p mimic
negative mimic
WT 3'UTR
MUT 3'UTR

Figure 4.13. Mir-211-5p is able to target and affect p63 expression. (A) QRT-PCR was used to validate the effectiveness of Pre-miR-211-5p treatment. Graphs show the fold change compared to Pre-miR control. **(B)** Cells either untreated (control), treated with a Pre-miR control or with Pre-miR-211-5p. Lysates were run on western blot and ΔNp63α protein expression was determined. GAPDH was used as a loading control**. (C)** Luciferase assay performed in HEK293 cells. Expression of human p63 3'UTR in a luciferase reporter gene (psi-check 2) leads to diminished luciferase activity in the presence of miR-211-5p. Plasmid containing mutant p63 3'UTR is included as a control. Error bars represent the SEM of three independent experiments performed in triplicate. Statistical analysis was performed using a two-tailed, unpaired Student's *t*-test. P-value ≤ 0.001 = ***.

4.2.11. Silencing of iASPP increases miR-211-5p levels

In section 4.2.10 data had shown miR-211-5p to be of importance to p63 regulation in the iASPP/p63 feedback loop. Confirmation of the effect of iASPP on miR-211-5p was therefore undertaken by qRT-PCR. As expected, silencing of iASPP resulted in a significant increase of miR-211-5p in IC4 (p=0.026) and IC18 (p=0.043) (Figure 4.14). Interestingly iASPP did not have an effect on miR-211-5p in N-TERT cells suggesting that this effect could be more specific to cancer cells.

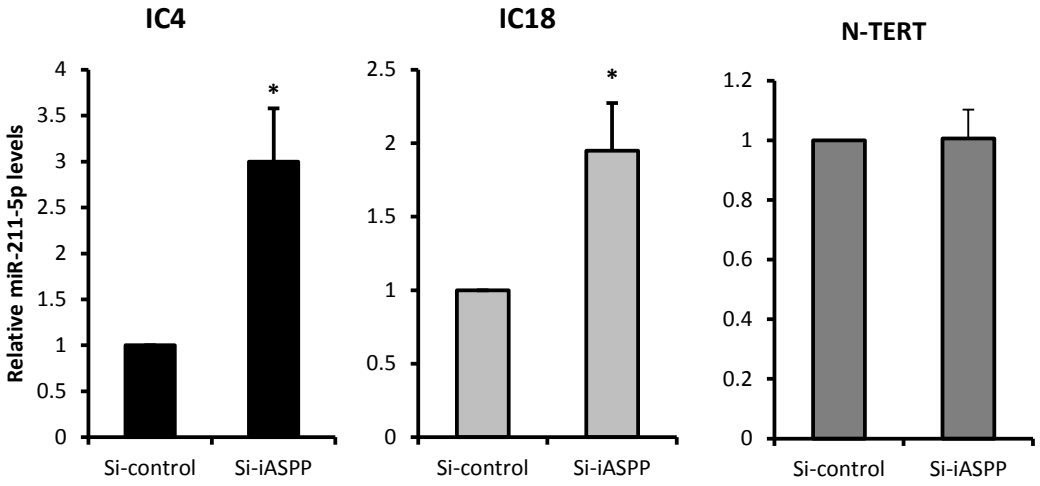


Figure 4.14. Validation of microRNA array by qRT-PCR. N-TERT and cSCC cells treated with si-control or si-iASPP (si-pool) were analysed by qRT-PCR for miR-211-5p expression. MicroRNA levels were normalised to internal control – RNU48. The fold change of si-iASPP compared to si-control is shown. Error bars represent the SEM for three independent experiments. Statistical analysis was performed using a two-tailed, unpaired Student's *t*-test, comparing the si-control to si-pool. P-value < 0.05 = *.

4.2.12. Overexpression of miR-211-5p is able to decrease the proliferation rate of the cell

Several studies have documented iASPP's physiological role in the cell. Chikh et al. (2011) found iASPP to be essential for cell proliferation in normal keratinocytes. A variety of studies demonstrate the importance of iASPP for proliferation in cancers including melanoma, oral squamous cell carcinoma, gastric, prostate, colorectal and bladder cancer, hepatocellular carcinoma, glioblastoma and NSCLC (Lu et al., 2010; Pang et al., 2010; Chen et al., 2010; Lin et al., 2011; Li et al., 2011; Zhang et al., 2011; Liu et al., 2011; Chen et al., 2014a; Chen et al., 2014b; liu et al., 2014; Pandolfi et al., 2015; Wang et al., 2015a). Silencing of iASPP causes cells to proliferate slower than control. It was hypothesised that cells containing unaltered expression levels of iASPP but overexpressing miR-211-5p would behave in the same way as cells silenced for iASPP. A timecourse was carried out prior to this to see for how long the transient transfection of Pre-miR was stable. IC4 and IC18 cells were transfected with Pre-miR-211-5p and samples were collected at 3, 5 and 7 days. Protein was extracted and cells were run on a 10% SDS-PAGE gel and blotted for ΔNp63α. Expression of ΔNp63α began to reappear at 7 days (Figure 4.15A). As a result a cell proliferation assay of up to 7 days was carried out on cells transfected with Pre-miR-211-5p. Cells were counted on days 3, 5 and 7. Both IC4 and IC18 cells transfected with Pre-miR-211-5p proliferated significantly slower than cells treated with Pre-miR-negative control (Figure 4.15B).

Colony forming assays measure the ability of individual cells to survive and proliferate. Previously, expression of iASPP has been demonstrated to be required for cells to produce colonies (Chikh et al., 2011; Zhang et al., 2011; Liu et al., 2011; Lin et al., 2011; Li et al., 2011; Liu et al., 2013a; Wang et al., 2015a). Cells overexpressing miR-211-5p were plated at a density of 1000 cells/plate and left to proliferate for 10 days. Although the effects of miR-211-5p appear to weaken at around 7 days, the initial transfection seemed sufficient in producing distinct results. After 10 days cells were fixed, stained with crystal violet and colonies of 50 cells or more were counted (Figure 4.15C) (Franken et al., 2006). Like the proliferation experiment it was hypothesised that cells containing unaltered expression levels of iASPP but overexpressing miR-211-5p would behave in the same way as cells silenced for iASPP. IC4 and IC18 cells overexpressing miR-211-5p formed significantly fewer colonies than cells expressing Pre-miR-negative control (IC4 $p=0.0038$; IC18 $p=0.005$). These data lead us to speculate that iASPP is able to control cell proliferation/colony formation via miR-211-5p. It is noted however that microRNA are often able to target several genes.

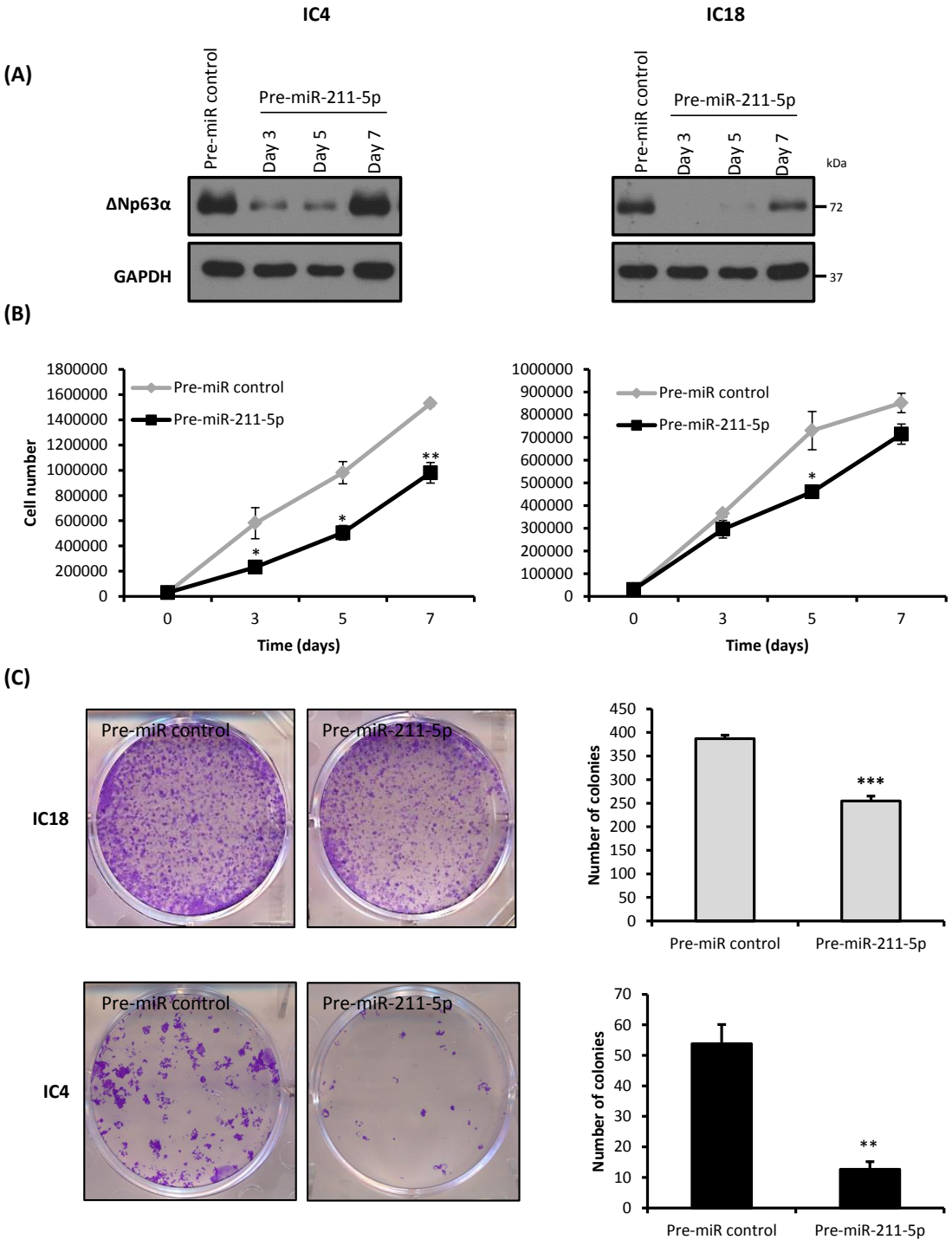


Figure 4.15. Pre-miR-211-5p treatment decreases the proliferation and colony forming ability of cSCC cells. (A) Time course showing the effect of cSCC Pre-miR-211-5p treated cells on ΔNp63α protein expression by western blot. **(B)** Growth curves of cSCC cells either treated with Pre-miR control or Pre-miR-211-5p analysed for their proliferation. **(C)** Representative images shown of colony forming assay. Graphs demonstrate the fold change difference of colony formation between cells treated with Pre-miR control or Pre-miR-211-5p. Error bars represent the SEM of three independent experiments

performed in triplicate. Statistical analysis was performed using a two-tailed, unpaired Student's *t*-test. P-value < 0.05 = *, ≤ 0.01 = **, < 0.001 = ***.

4.3. Summary

The aim of this project was to dissect the role of iASPP, a novel crucial regulator of epidermal homeostasis, in keratinocyte skin carcinogenesis. I wanted to investigate if the novel p63/iASPP feedback loop discovered by Chikh et al. (2011) was maintained or dysregulated in cSCC. I hypothesised that this feedback loop would be altered in cancer.

In this chapter it was discovered that the p63/iASPP axis was still intact in cSCC with silenced p63 able to downregulate iASPP at the protein and mRNA level and silenced iASPP in turn affecting the protein expression of p63 but not mRNA levels. MicroRNA array data enabled us to further investigate the inability of iASPP to downregulate p63 at solely the protein and not mRNA level. This led us to identify the importance of miR-211-5p expression, a microRNA that was predicted to target p63 but that had no published data on its actual ability to target p63. Research into the function of miR-211-5p in other cancers had provided conflicting evidence; however the role of miR-211-5p in cSCC has not been studied. Investigation into miR-211-5p provided evidence that iASPP physiological effects on the cell may be signalled at least in part through miR-211-5p (Figure 4.16).

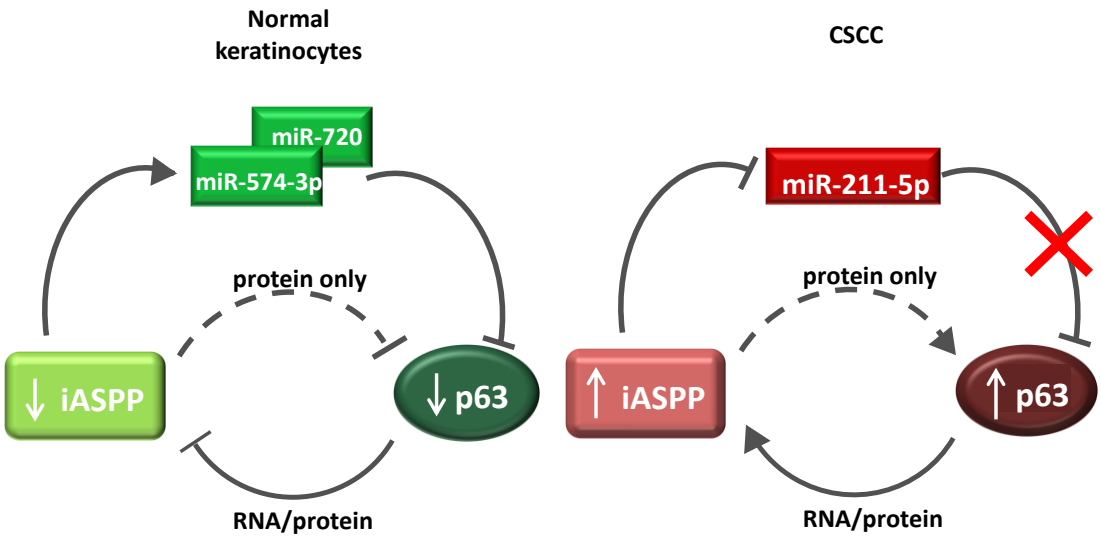


Figure 4.16. iASPP/p63 autoregulatory feedback loop. In normal keratinocytes miR-574-3p and miR-720 are able to control the expression of p63 and in turn iASPP. In cSCC miR-211-5p is able to control the expression of p63. High levels of iASPP in cSCC result in the downregulation of miR-211-5p and, in turn, lack of p63 inhibition.

In this project, I have stained and scored human cSCC specimens and found high p63 expression levels and high cytoplasmic/nuclear iASPP expression in the tumours. In support of this finding I have also screened a panel of 10 cSCC cell lines confirming high expression of iASPP and ΔNp63. Three cSCC cell lines with differing UV/mutation statuses were selected for further study: IC4, IC15 and IC18. Knockdown of p63 reduced the protein expression and mRNA levels of iASPP in cSCC cell lines. Consistent with these results, knockdown of iASPP reduced p63 protein expression. p63 mRNA levels remained the same when iASPP was silenced, as was observed in N-TERT cells by myself and Chikh et al. (2011), suggesting an extra layer of control in the feedback loop when iASPP is silenced.

In HaCaT cells the extra layer of control between iASPP and p63 had come from microRNA, miR-574-3p and miR-720. In cSCC the levels of these microRNA were examined by screening our cSCC cell line panel and collaborating with colleagues in Germany to perform *in situ* hybridisation on cSCC tissue. These data showed us that miR-574-3p levels were decreased in our cell line panel and also had lower levels of expression observed in cSCC tissues compared to the normal skin sections. Levels of miR-720 were also reduced in cSCC tissues compared to normal skin; however, an increase was observed when screening cSCC cell lines. Although there is increasing literature characterising the role of miR-720, linking it with cell differentiation and tumour metastasis and it appears to behave in many contexts as a microRNA, we had noticed while performing our research that miR-720 was no longer classed as a microRNA but was now recognised as a tRNA (Schopman et al., 2010).

MicroRNA are in control of p63 in HaCaT cells. In cSCC when p63 is overexpressed and microRNA levels are downregulated, at least in the tissue, this suggests that perhaps these microRNA are no longer controlling p63 in cSCC. To investigate this further we used anti-miR inhibitors but with limited success; it is possible that trying to inhibit already low levels of microRNA has a minimal effect on high levels of p63. What was successful though was the addition of pre-miR microRNA mimics in cSCC to decrease p63 expression. This confirmed that an artificial increase of microRNA is still sufficient to control p63 expression. We also investigated whether iASPP was still in control of miR-574-3p. In cSCC cell lines silenced for iASPP we did not observe an increase in microRNA levels suggesting that iASPP is no longer in control of the miR-574-3p. At present the above microRNA data remain to be validated by replication of the original findings in N-TERT cells. A plausible reason to explain difficulty in

reproducing this finding could be that the previous data was only performed in HaCaT cell lines stably knocked down for iASPP (therefore using another silencing sequence).

MicroRNA array, luciferase assays and overexpression data in IC4 and IC18 cells silenced for iASPP provided a new target for iASPP and a novel regulator of p63 in cSCC – miR-211-5p. N-TERT cells did not have this upregulation of miR-211-5p suggesting a cSCC-specific phenomenon. Interestingly, however, IC15 appeared to have a very different microRNA profile compared to IC4 and IC18, this is likely due to the fact that IC15 is HPV-16 induced and not UV-associated. Further analysis of miR-211-5p demonstrated its effects on proliferation and colony forming ability. iASPP had previously been shown to promote the proliferation and colony forming ability of cells of different origins. Overexpressing miR-211-5p demonstrated similar effects to silencing iASPP, hinting at the fact that iASPP may be signalling through miR-211-5p. This could, however, just be because of the effect of miR-211-5p on p63. Further research showing that iASPP is actually able to interact with miR-211-5p would need to be carried out to strengthen this research.

In the previous chapter it was noted that the location of iASPP in the cell appeared to differ between tumour sections and this was investigated further in our panel of cSCC cell lines. The immunofluorescence and subcellular fractionation cell line data supported the observation that iASPP appeared to be mainly cytoplasmic and p63 nuclear in the cSCC cell lines and tumours. Reflecting on these results and data showing the p63/iASPP axis is still intact, a subcellular fractionation was performed with cSCC cells silenced for p63. Results show that p63 is able to affect the expression of iASPP regardless of whether it is nuclear or cytoplasmic. It is plausible that iASPP is affected by p63 in the nucleus and then shuttled out to the cytoplasm. However, further work would be required to investigate this.

Research into the status in cSCC of a regulatory feedback loop responsible for maintaining cell homeostasis in human keratinocytes was undertaken with the hope of discovering a new biomarker and/or pathway to target therapeutically. Demonstrating that miR-211-5p is potentially downregulated in cSCC provides novel data on the molecular basis of squamous carcinogenesis. In this chapter I have discovered a novel way to reduce the expression of oncogenic p63 and additionally affect the downstream effects of the feedback loop in cSCC. By targeting miR-211-5p this can be done without altering the levels of iASPP, a protein which is essential for cell homeostasis. Although still in the early stages of research, the miR-211-

5p could have the potential to become a target for cSCC therapy. Additionally, the discovery that miR-211-5p is not affected by iASPP in N-TERT cells leads me to speculate that involvement of miR-211-5p in cSCC is an exclusive event and therefore makes it a promising target for the future.

Chapter 5: Pathophysiological effects of iASPP in cSCC

5.1. Introduction and aims

The effects of iASPP on cell homeostasis in the skin have recently been investigated. Chikh et al. (2011) found that iASPP played a role in regulating epidermal adhesion and proliferation whereby depletion of iASPP in HaCaT cells triggered a deregulation of the cell junctional complex integrity. Upon the silencing of iASPP, several genes involved in cell-matrix adhesion, for example β1 integrin, desmosmal and adhesion proteins including PERP and Claudin 1, amongst others, were down regulated. iASPP is implicated in the differentiation and stratification of the epidermis and iASPP depletion results in an increased epidermal thickness. This observation was correlated with an increase in the expression of both involucrin and loricrin, markers of keratinocyte terminal differentiation (Chikh et al., 2011). The latter effect has been further confirmed by a report using an iASPP knockout mouse (Notari et al., 2011). In the same study the authors also reported that in mouse embryonic fibroblasts, iASPP was able to inhibit premature senescence in cells (Notari et al., 2011).

Silenced iASPP causes a negative effect on HaCaT cell proliferation due to delayed cell-cycle progression, with a reduction in cyclin D2 expression. In support of this effect on proliferation, a decline in the number of colonies formed upon treatment of HaCaT cells with shRNA against iASPP was observed (Chikh et al. 2011). Several papers also support a role for iASPP controlling cell proliferation in a cancer setting. Depletion of iASPP in prostate, bladder and gastric cancer cell lines in addition to non-small cell lung cancer (NSCLC), glioblastoma and hepatocellular carcinoma cells causes a negative effect on the cells ability to proliferate (Pang et al., 2010; Lu et al., 2010; Zhang et al., 2011; Lin et al., 2011; Chen et al., 2011; Li et al., 2011; Lu et al., 2013; Morris et al., 2014; Wang et al., 2015a). In melanoma the HEDGEHOG/GLI-E2F1 axis positively modulates iASPPs ability to regulate cell proliferation (Pandolfi et al., 2015). Chen et al. (2014b) showed the negative effect iASPP had upon depletion from oral tongue squamous cell carcinoma cell lines. However, no reports to date have documented the effects of iASPP on cell proliferation in cSCC.

In prostate, glioblastoma and colorectal cancer cell lines the reduction of cell proliferation upon iASPP silencing was directly linked to miR-124 (Zhao et al., 2013; Chen et al., 2014a; Liu

et al., 2014). Luciferase assays confirmed the direct interaction of miR-124 with iASPP. Overexpression of miR-124 in prostate, glioblastoma and colorectal cancer cell lines had the same effect on proliferation as silencing iASPP. Interestingly in cSCC, the expression of miR-124 was significantly downregulated both *in vitro* and *in vivo* (Yamane et al., 2013).

Studies have demonstrated the role of iASPP as both a p53-dependent and p53-independent inhibitor of apoptosis (Bergamaschi et al., 2003; Cai et al., 2012b). Breast cancer cells containing wild type p53 show increased UV and cisplatin-induced apoptosis when treated with siRNA against iASPP (Bergamaschi et al., 2003). iASPP is able to inhibit p53-induced apoptosis by binding to p53 and preventing its transcriptional activity on pro-apoptotic BAX and PIG3 promoters (Bergamaschi et al., 2003). The overexpression of iASPP in NSCLC cells deficient of p53 was still able to trigger apoptosis by inhibiting pro-apoptotic transcriptional activity of p63 and p73 (Cai et al., 2012b). Although a well-documented inhibitor of apoptosis in a cancer setting, conflicting studies also support the notion presented by Laska et al. (2007) that iASPP does not always play an anti-apoptotic role (Pinto et al., 2010; Chikh et al., 2014). Laska et al. (2007) demonstrated that in non-transformed cells silenced for iASPP and treated with etoposide there was a reduction of apoptosis. Chikh *et al*., (2014) have demonstrated another novel function of iASPP in non-transformed cells showing that silenced iASPP in non-transformed keratinocytes did not cause an increase in apoptosis but rather triggered an increase in autophagy, providing a role for iASPP as a novel autophagy inhibitor. Studies showing a reduction of apoptosis upon iASPP depletion in the cell are not solely limited to non-transforming cells. Pinto et al. (2010) speculate that iASPP may have a dual role in apoptosis in precancerous lesions, specifically, pituitary tumours. More recently, in melanoma iASPP displays a pro-apoptotic phenotype whereby treatment of melanoma cell lines, depleted for iASPP, with cisplatin causes a decrease in apoptosis through the interaction of iASPP with acetyltransferases p300 and CBP, which are able to acetylate and thus stabilize both p53 and TAp73. The activation and stabilization of both p53 and TAp73 allows the transcriptional upregulation of pro-apoptotic genes (Kramer et al., 2015).

Further research into other physiological effects of iASPP in a cancer setting has been very limited. At present, to the best of my knowledge, only a handful of studies have touched upon the effect of iASPP on the invasive potential of the cell. In addition, these studies have been observational studies reporting correlations between iASPP expression and invasive tumour tissue/metastasis (Liu et al., 2010; Cao et al., 2013; Kim et al., 2015b). At this time

there appears to be a lack of functional studies that investigate the actual role of iASPP in cancer. This is important research that needs to be carried out to enable a better understanding of why iASPP is so highly expressed in cancer and to provide ways of manipulating iASPP or its regulators/binding partners from a therapeutic perspective. Therefore the aims of this chapter are to:

1) Investigate the role of iASPP on the proliferation of cSCC cell lines
2) Discover whether iASPP plays a pro-apoptotic or anti-apoptotic role in cSCC cell lines
3) Explore the effect of iASPP on the cells potential to invade/migrate
4) Examine the pathways involved in these effects

5.2. Results

5.2.1. iASPP is essential for cSCC proliferation

The effect of iASPP on cell proliferation has been well documented in both the normal skin and various types of cancer (Pang et al., 2010; Lu et al., 2010; Zhang et al., 2011; Lin et al., 2011; Chen et al., 2011, Li et al., 2011; Chikh et al., 2011; Notari et al., 2012; Lu et al., 2013; Zhao et al., 2013; Chen et al., 2014b; Liu et al., 2014; Morris et al., 2014; Pandolfi et al., 2015; Wang et al., 2015a). However to date, no studies have been performed analysing the effects of iASPP on cell proliferation in cSCC. Based on the previous research on the skin and other tumour types, it was hypothesised that iASPP would have a positive effect on cell proliferation.

To investigate the role of iASPP on proliferation in cSCC cells, a growth curve was carried out comparing IC4, IC18 and IC15 cells silenced for iASPP. These three cSCC cell lines were used as a continuing model to study cSCC due to the differing mutational status and origin of the cells (Table 4.1). To enable the investigation into the role of iASPP on cell proliferation in cSCC, iASPP had to be silenced for enough time to allow the experiment to be carried out. To determine how long iASPP knockdown was effective for I carried out a time course experiment. cSCC cell lines were transfected with 50nM siRNA targeting iASPP and cells were pelleted on days 3, 5, 7 and 9. Cell lysates were run on a western blot and membranes were probed with an iASPP antibody (Figure 5.1). In the IC15 and IC18 cell lines iASPP was efficiently knocked down until day 9. The IC4 cell line was silenced until day 7, after this iASPP expression began to reappear.

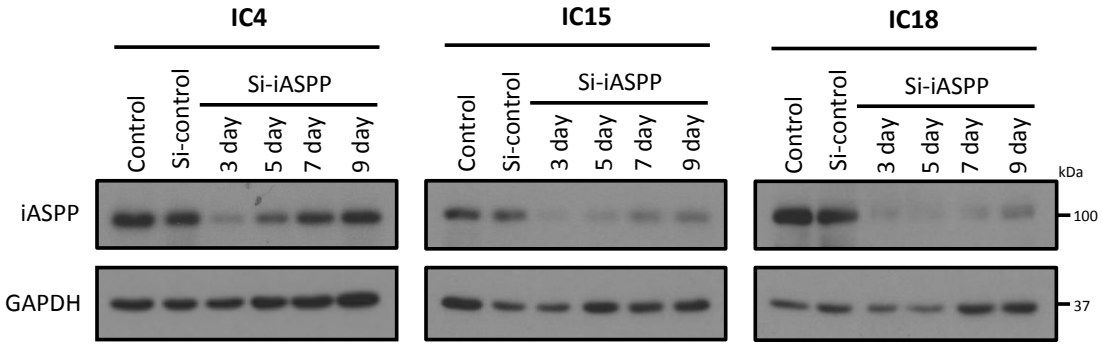


Figure 5.1. Timecourse of iASPP knockdown in cSCC cells. Western blot showing the duration of iASPP knockdown (si-iASPP – si-pool) compared to control and si-control over a total of 9 days in three different cSCC cell lines.

Using the silencing efficiency as a guideline, a proliferation assay was undertaken for 7 days. Cells were plated in triplicate wells, transfected with si-iASPP and si-control and then counted using a haemocytometer on days 3, 5 and 7. The data show that when iASPP is silenced, cells proliferated at a slower rate compared to si-control, demonstrating that, in these cells, iASPP is also required for the proliferation of cancer cells (Figure 5.2). Prior to undertaking these experiments, proliferation rates of cells transfected with si-control were compared to untreated cells to ensure no effects of si-control on proliferation.

Chikh et al. (2011) had previously shown similar growth curves in HaCaT cells. Further support for this decrease in proliferation detected in HaCaT cells depleted for iASPP came from a bromodeoxyuridine kinetic analysis showing that iASPP was able to alter cell-cycle progression delaying entry into G1 phase. This was accompanied by a downregulation of cyclin D2 (Chikh et al., 2011). Cyclin D2 is required to complex with CDK4/6 to enable the cell to cycle through G1/S phase. Western blots performed with cSCC cell lysates silenced for iASPP show a downregulation of cyclin D2 expression levels supporting the previous findings in HaCaT cells and transferring them to a tumour setting (Figure 5.3).

In the previous chapter I have shown that iASPP and p63 are controlled by an autoregulatory feedback loop, thus silencing iASPP leads to a decrease in p63 expression. ΔNp63 also regulates proliferation (Sbisà et al., 2006). The positive role of ΔNp63 regarding proliferation, taken together with the positive role for iASPP regarding proliferation found in this project, suggest that the feedback loop as a whole contributes to regulating cell proliferation (Sbisà et al., 2006).

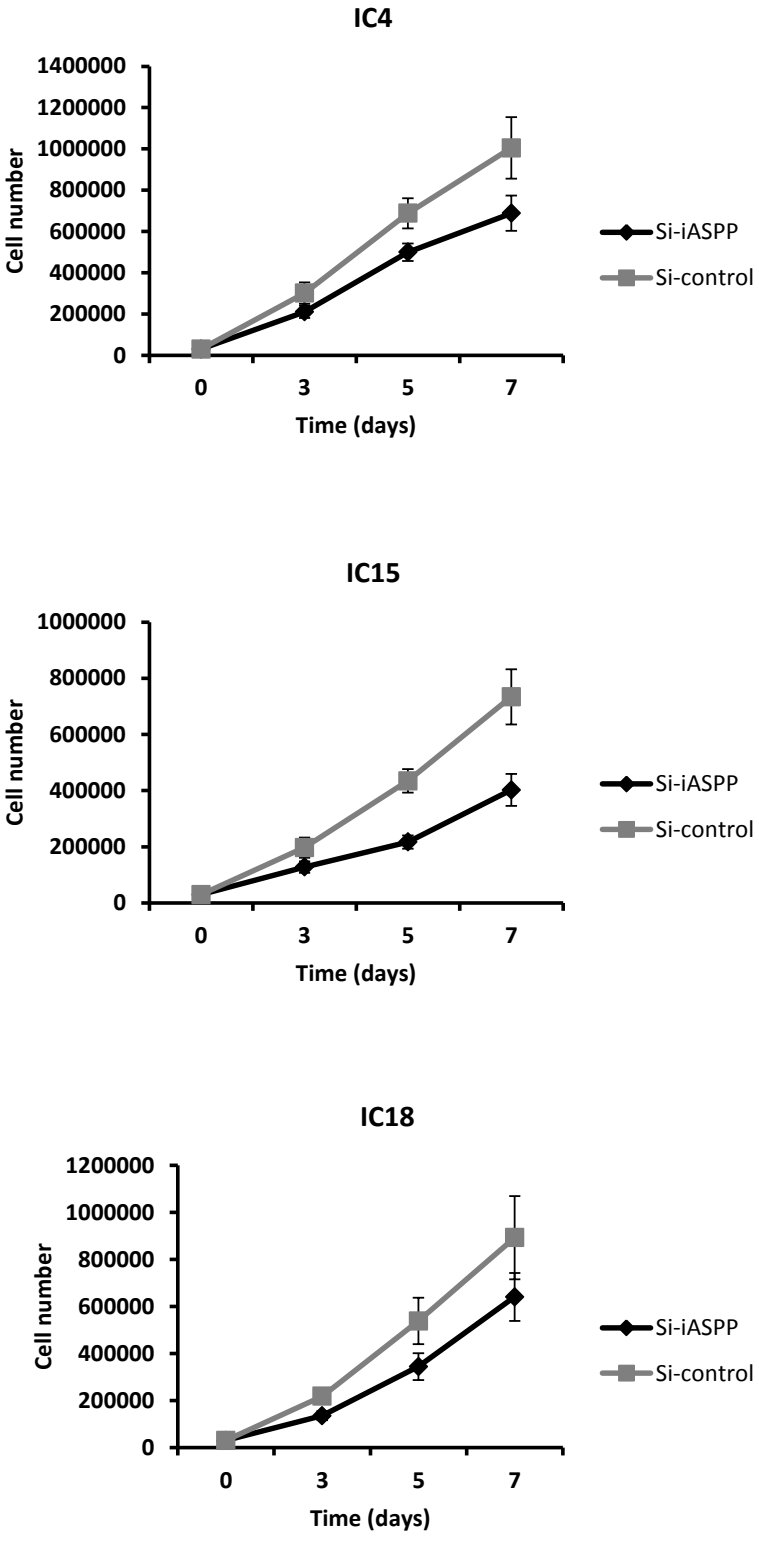


Figure 5.2. Expression of iASPP affects the proliferation of cSCC cell lines. Growth curves of three individual cSCC cells treated with si-control or si-iASPP (si-pool) analysed for their proliferation over 7 days. Error bars represent the SEM of three individual experiments performed in triplicate. Statistical analysis was performed using a two-tailed, unpaired Student's *t*-test – no statistical significance was found.

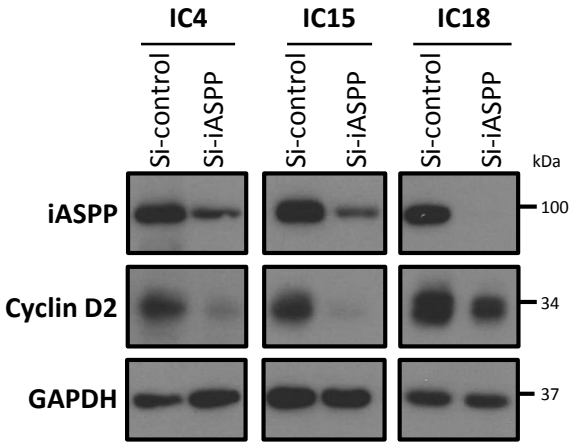


Figure 5.3. iASPP silencing decreases cyclin D2 expression. Western blots show the effect of si-iASPP (si-pool) on cyclin D2. Cells were harvested 72 hours after transfection. GAPDH was used as a loading control.

The effect of iASPP on proliferation was also investigated using a colony forming assay. A colony forming assay determines the ability of a cell to grow into a colony. Colony forming assays were carried out for 10 days in IC4, IC15 and IC18 cell lines transfected with either si-iASPP or si-control. Cells were seeded at a density of 1000 cells per well in a 6-well plate (Figure 5.4A). After 10 days cells were fixed. Colonies of 50 cells or more were counted. Initially a total of 50 cells were counted and the size of this colony was noted, from then on colonies of a similar size were tallied (Franken et al., 2006). Although iASPP expression in the IC4 cell line in particular started to reappear at 7 days, the effects of iASPP knockdown were still able to be visualised. In all three cSCC cell lines, cells depleted for iASPP show a reduction in the number of colonies formed. In the case of IC4 and IC15 a significant reduction (Figure 5.4B) (IC4 $p= 0.05$, IC15 $p= 0.02$) was observed.

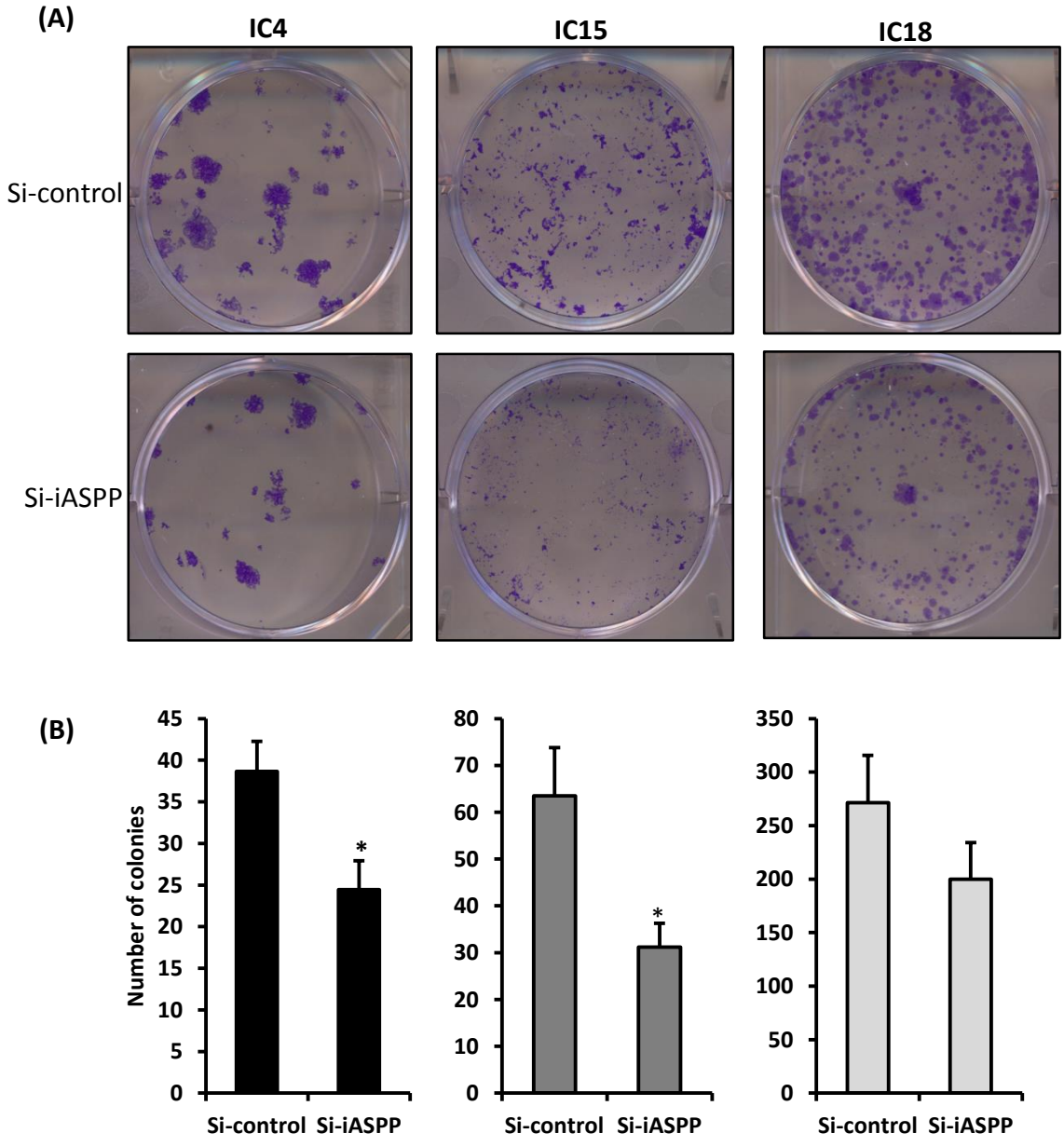


Figure 5.4. Knockdown of iASPP reduces the colony forming ability of cSCC cells. (A) Representative images shown of colony forming assay in three cSCC cell lines. Purple staining represents cell colonies. **(B)** Graphs show the difference in the number of colonies formed between cells treated with si-control and cells treated with si-iASPP after 10 days (si-pool). Error bars represent the SEM of three independent experiments performed in triplicate. Statistical analysis was performed using a two-tailed, unpaired Student's *t*-test, comparing the si-control to si-pool. P-value ≤ 0.05 = *.

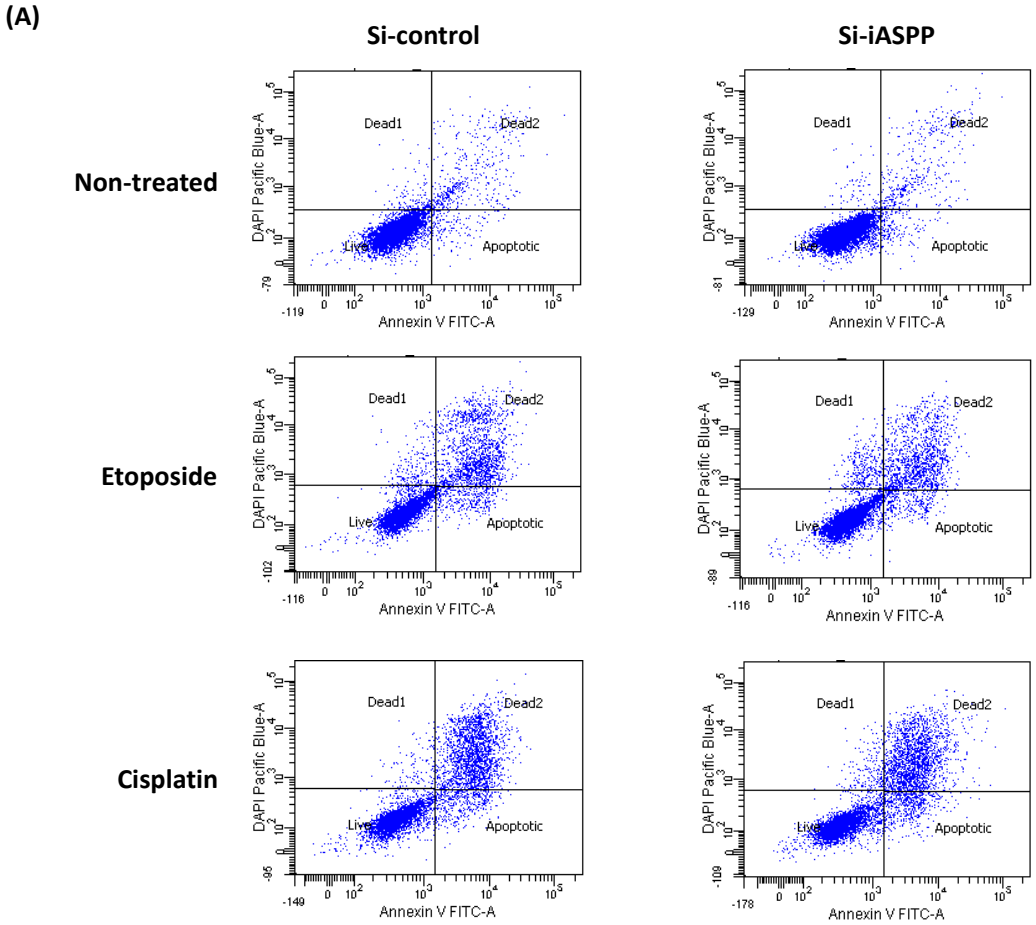
5.2.2. Investigation into the dual role of iASPP in apoptosis

It has been well documented that iASPP can act as an inhibitor of apoptosis in cancer cells. However a handful of studies have reported a pro-apoptotic function of iASPP in both non-transformed cells and precancerous lesions but also more recently in cancerous cells (Laska et al., 2007; Pinto et al., 2010; Kramer et al., 2015). Alongside the multiple roles of iASPP reported so far, recent data also demonstrated a role for iASPP as an autophagy inhibitor in the normal skin. I therefore wanted to investigate the role of iASPP in apoptosis in cSCC to determine if iASPP's role was cell type specific (Chikh et al., 2014).

Apoptosis was induced in cSCC cell lines using etoposide and cisplatin. These two chemotherapeutic drugs were chosen due their use in apoptotic models in previous studies involving iASPP (Bergamaschi et al., 2003; Liu et al., 2009; Kramer et al., 2015). Etoposide functions by binding to topoisomerase II and causes breakage of DNA strands leading the cells to apoptose (van Maanen et al., 1988). Cisplatin functions by crosslinking the DNA causing the cells to undergo apoptosis (Siddik 2003). Based on evidence from the majority of past cancer cell studies, it was predicted that there would be a significant increase in apoptosis upon iASPP silencing. To assess the effects of iASPP on apoptosis an Annexin-V-FITC assay was used and cells were analysed by flow cytometry. Cells undergoing apoptosis expose phosphatidylserine on the surface of the cell that acts as a signal for the cell to be phagocytosed (Verhoven et al., 1995). Annexin-V is able to bind to exposed phosphatidylserine and thus is used as a marker for apoptosis. Cells were co-stained with DAPI, a fluorescent cell viability marker. DAPI is taken up by dead cells at a higher efficiency than live cells. The apoptotic cell population included Annexin V-FITC positive, DAPI negative and Annexin V-FITC positive, DAPI positive (right upper and lower quadrants of scatter plot; Figure 5.5A). Cells were treated with either si-control or si-iASPP prior to the addition of etoposide, cisplatin or no drug (non-treated). IC4, IC15 and IC18 cells were analysed. The majority of the literature to date describes the apoptotic role of iASPP as p53-dependent. To take into account any differences that may be observed due to the mutation status of p53, cell lines with both wild type and mutated p53 were included (Table 4.1). Representative scatter plots of IC4 are shown alongside graphs depicting the percentage of apoptotic cells (Figure 5.5A).

cSCC cells silenced for iASPP and either untreated or treated with etoposide or cisplatin did not appear to have an increase in apoptosis (Figure 5.5B). Instead the levels of apoptosis

remain the same in si-iASPP treated cells compared to si-control treated cells or conversely become slightly decreased, though this was not significant. Chikh et al., (2014) have demonstrated that silenced iASPP in non-transformed keratinocytes did not cause an increase in apoptosis but rather triggered an increase in autophagy providing a role for iASPP as an inhibitor of autophagy. Perhaps cells of different lineages can behave differently with regard to iASPP.



(B)

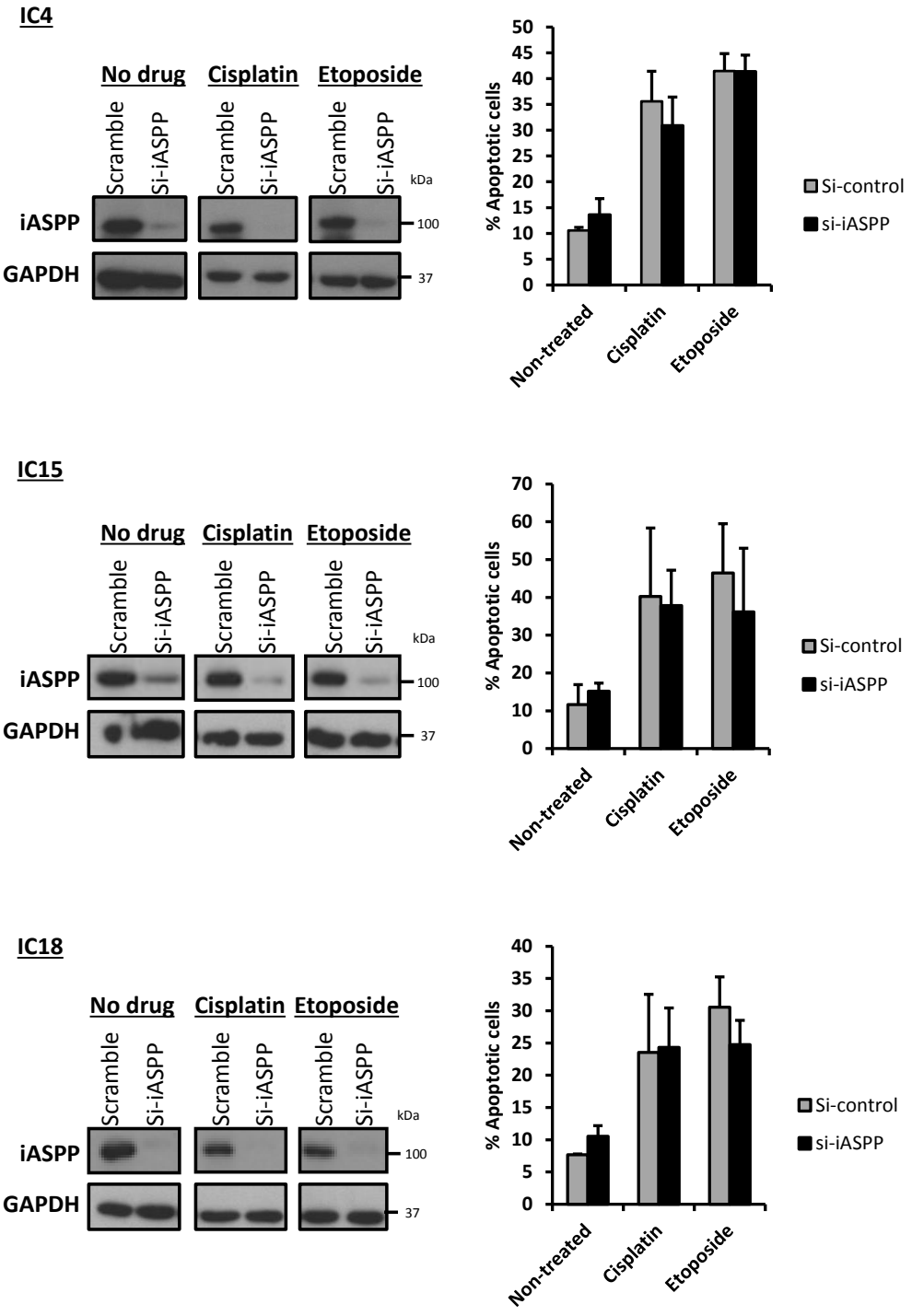


Figure 5.5. Knockdown of iASPP does not appear to increase apoptosis in etoposide/cisplatin-treated cells. (A) Representative scatter plots from IC4 cell line transfected with si-iASPP (si-pool) and si-control and treated with cisplatin and etoposide. Scatter in the top right quadrant (dead2) was classed as late apoptotic, the bottom right quadrant (apoptotic) was classed as early apoptotic, the top left quadrant (dead1) was classed as cell debris and the bottom left quadrant (live) was classed as live cells. **(B)** Three cSCC cell lines transfected with si-control and si-iASPP (si-pool) treated with etoposide or cisplatin for 18 h analysed by FACS. The apoptotic cell percentage is a combination of the

cells undergoing early and late apoptosis. Error bars represent the SEM of three independent experiments. Western blots show effective iASPP knockdown at the time of the experiment. GAPDH was used as a loading control.

To investigate the role of iASPP with regard to apoptosis and cSCC further, I wanted to investigate whether depleting iASPP in cSCC was actually promoting autophagy. As mentioned above, using a combination of N-TERT, HaCaT and primary keratinocyte cells silenced for iASPP, no increase in apoptosis was observed. Instead keratinocytes silenced for iASPP displayed increased autophagy providing a role for iASPP as an inhibitor of autophagy (Chikh et al., 2014). To investigate this I ran cSCC cell lines treated with si-control and si-iASPP on a western blot and probed with an LC3 antibody. Additionally N-TERT cells both treated with si-control and si-iASPP were included as a positive control. When autophagy is induced, LC3-I becomes lipidated producing LC3-II (Kabeya et al., 2000). Although LC3-II has a higher molecular weight than LC3-I, LC3-II migrates more rapidly on an SDS–PAGE gel likely due to higher hydrophobicity associated with the phosphatidylethanolamine group to which it is conjugated (Barth et al., 2010). Consistent with Chikh et al. (2014) figure 5.6 shows that N-TERT cells silenced for iASPP display higher levels of LC3-II compared to control. Interestingly, none of the cSCC cells depleted for iASPP showed higher levels of LC3-II lipidation. These data suggest that in cSCC iASPP may not be an inhibitor of autophagy. Of note, however, are the high levels of LC3-II and thus autophagy already occurring in non-silenced cSCC cells. A study into cSCC found an increased resistance of advanced stage cSCC cells to cisplatin treatment. Further investigation into this observation found that along with high levels AKT, high levels of autophagy were occurring in advanced cSCC, measured by high accumulation of LC3-II. Treatment of these resistant cells with AKT inhibition alongside autophagy inhibitor, 3-methyladenine, enhanced the cytotoxicity of cisplatin (Claerhout et al., 2010).

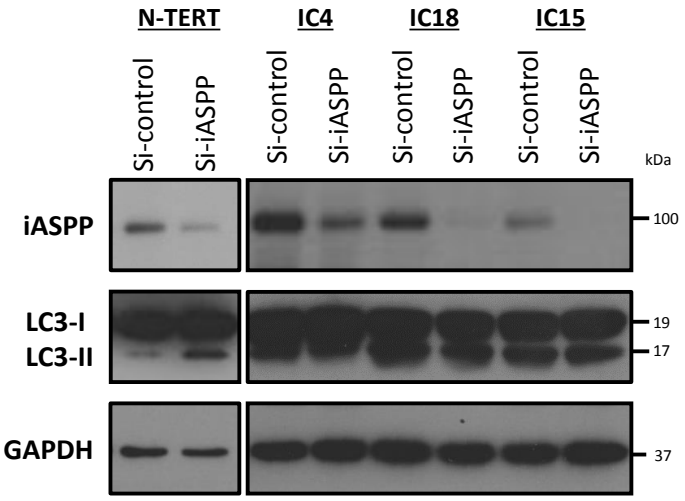


Figure 5.6. In cSCC iASPP does not appear to be an inhibitor of apoptosis. N-TERT and three cSCC cell lines were depleted of iASPP (si-pool). Western blots were run and probed for LC3-I and LC3-II, a marker of autophagy. GAPDH was used as a loading control.

5.2.3. Silencing iASPP increases cell motility and migration

Although there have been numerous reports on the oncogenic role of iASPP in cancer, to date there have been no reports investigating the role of iASPP on cell migration/motility. A couple of papers have detailed the importance of iASPP in adhesion, although these have been carried out in non-cancerous cell models (Chikh et al., 2011; Notari et al., 2015). iASPP is integral in maintaining cell junctions. iASPP deficient mice develop arrhythmogenic right ventricular cardiomyopathy (ARVC) known to be caused by defects in desmosome integrity. Additionally, desmosomal complexes in HaCaT cells silenced for iASPP become dysregulated. Reduction in cell adhesion has been reported in many cancers and this reduction is correlated with increased cell migration and metastasis. It was interesting that the loss of iASPP expression caused defects in desmosomal integrity, a marker of cell invasion and metastasis. However, iASPP is predominantly regarded as an oncogene. Due to these conflicting ideas I wanted to investigate whether the loss of adhesion previously observed in cells depleted for iASPP was able to affect the motility of the cell.

To investigate this, IC4, IC15 and IC18 cell lines, transfected with si-iASPP and si-control, were tracked using Metamorph Image Analysis Software. Each different experimental condition was performed in duplicate. Three time-lapse videos were taken per well and within those three images, 10 cells were tracked over 8h. Figure 5.7A shows a representative still image from an IC15 time-lapse video. This image shows the striking difference between cells treated with si-control compared to cells treated with si-iASPP. Cells treated with si-control adhere to each other forming tight cell colonies. In contrast, cells depleted for iASPP appear to form fewer cell-cell contacts and therefore fewer colonies. Metamorph Image Analysis calculated the mean velocity of each cell that was tracked. Cells silenced for iASPP in IC4 cell line had a significantly higher mean velocity that cells treated with si-control (Figure 5.7B) ($p=0.005$). Although the IC15 and IC18 mean velocity data was not significant they too displayed the same trend as IC4 showing an increase in mean velocity when depleted of iASPP. The apparent loss of cell-cell contacts in Figure 5.7A appears to cause the cell to become more motile.

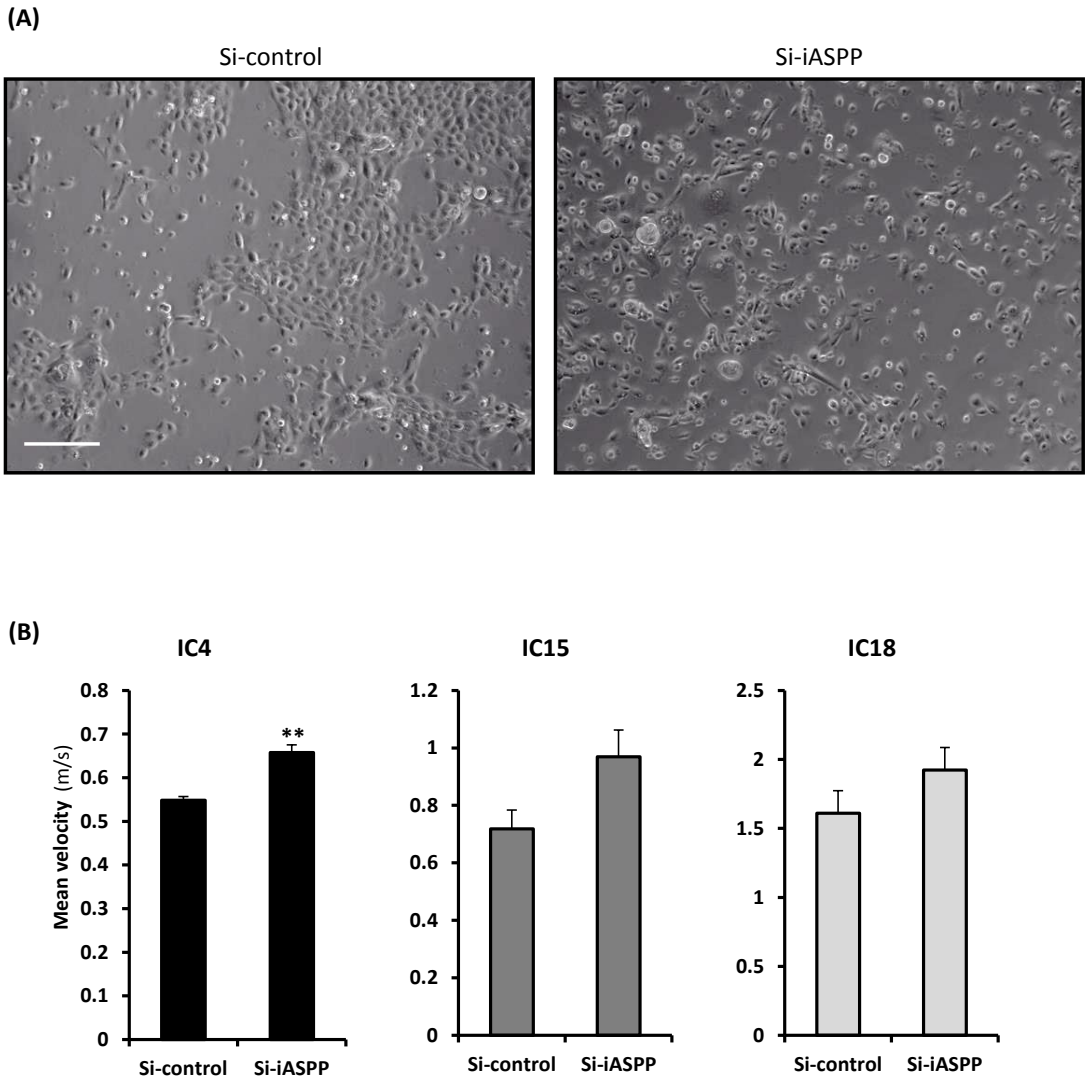


Figure 5.7. Silenced iASPP can affect cell motility in cSCC. (A) Representative images from IC15 cell line silenced for iASPP and control. Scale bar represents 100 µm **(B)** Graphs demonstrate the mean velocity of three cSCC cell lines treated with si-control and si-iASPP (si-pool). Error bars represent the SEM of three independent experiments. Statistical analysis was performed using a two-tailed, unpaired Student's *t*-test, comparing the si-control to si-pool. P-value ≤ 0.01 = **.

To investigate this increase in cell motility further I silenced two cSCC cell lines, IC4 and IC15 for iASPP and measured the effect of iASPP on cell migration using transwell inserts (Figure 5.8A&B) (Biddle et al., 2011). Cells were plated on the top of the inserts in medium containing 2% FBS and left for 24 h to migrate through to the bottom of the well containing 10% FBS. Cells that had migrated through to the underside of the insert were fixed, stained with crystal violet and counted. In agreement with the hypothesis, the loss of adhesion in cells silenced for iASPP published previously, correlates with the finding that cSCC cells silenced for iASPP were able to migrate more than non-silenced cells. cSCC cells transfected with si-iASPP were able to migrate significantly more than cells treated with si-control (IC4 $p=0.031$, IC15 $p=0.045$). Consistent with these findings, loss of ΔNp63α in SCC cell lines promoted cell migration and increased cell motility (Barbieri et al., 2006). These data provide evidence that it is not iASPP alone, but the autoregulatory feedback loop as a whole, that is able to control cell migration and motility.

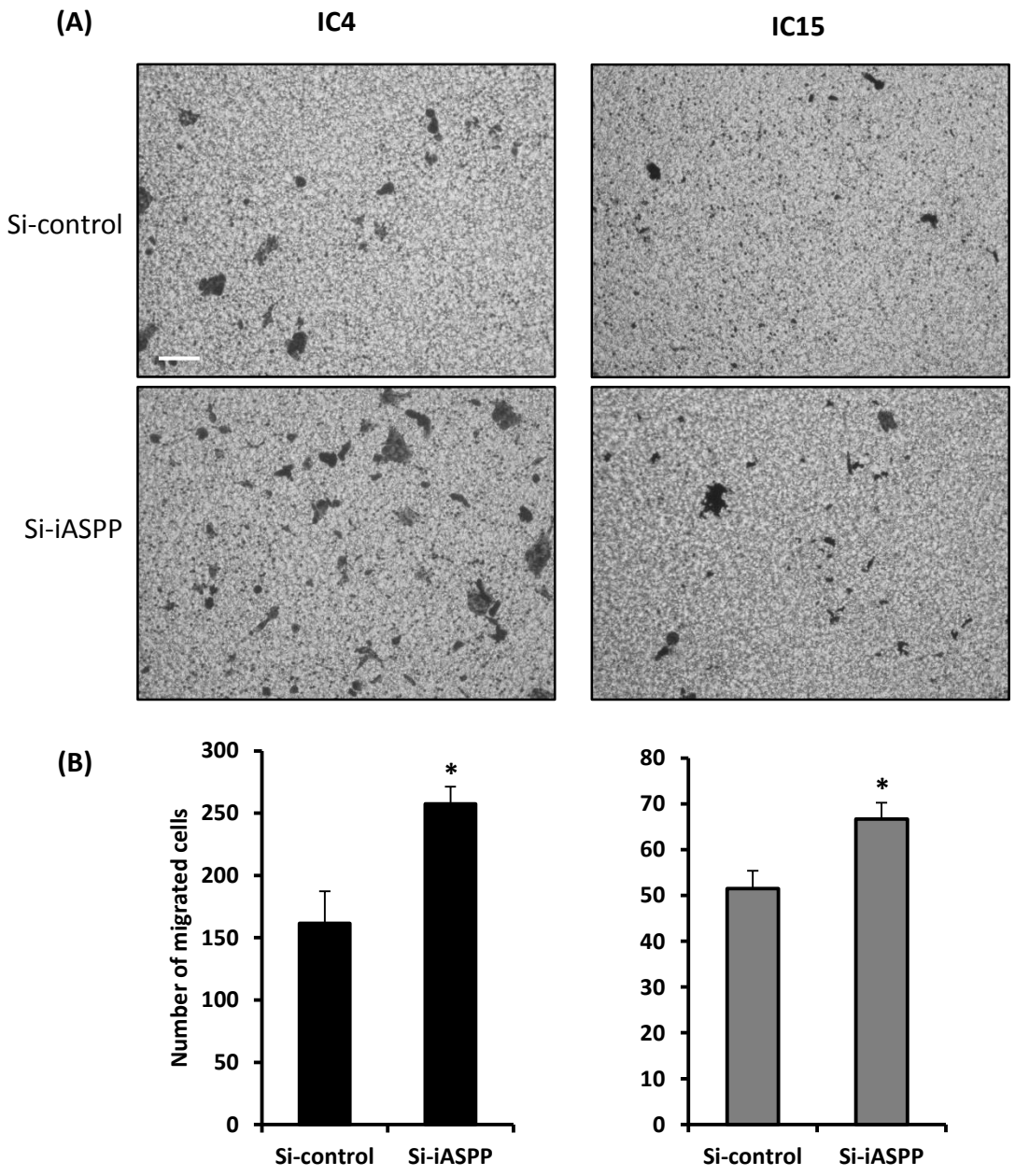


Figure 5.8. Silencing of iASPP affects the migration of cSCC cells. (A) Representative images of two cSCC cell lines, IC4 and IC15, treated with si-control or si-iASPP (si-pool) undergoing migration. Scale bar represents 100 µm **(B)** Graphs show the number of cells migrated in si-control and si-iASPP (si-pool) treated cells. Error bars represent the SEM of three independent experiments performed in duplicate. Statistical analysis was performed using a two-tailed, unpaired Student's *t*-test, comparing the si-control to si-pool. P-value ≤ 0.05 = *.

5.2.4. Role of iASPP in invasion

In order to investigate in more detail the effects of increased cell motility and migration upon depletion of iASPP, 3D organotypic cultures were produced. Organotypic cultures are 3D *in vitro* models used to investigate the invasion of tumour cells. Here, using cSCC cell lines, we were able to mimic the formation of cSCC tumours. The aim was to investigate the effect of iASPP on cell invasion. Organotypic cultures were produced using a combination of Collagen I and Matrigel® Basement Membrane Matrix in order to mimic *in vitro* the epidermis of the skin (Fusenig et al., 1983; Nystrom et al, 2005). Stromal cells are essential for a cancer cell to invade. Thus, in this case, human foreskin fibroblasts are included to help mimic the *in vitro* epidermis and also promote invasion of cancer cells into the dermis (Lewis et al., 2004). Organotypic cultures are typically left to incubate for 10-14 days. Due to the lack of success using shRNA as a silencing technique for iASPP (Chapter 4), IC4 and IC15 cells were treated with both si-control and si-iASPP. As observed in Figure 5.1, in IC15 cells at 9 days iASPP knockdown was still effective. However in the IC4 cell line iASPP expression began to reappear at 7 days. For this reason I chose to incubate the organotypics for the shorter time point of 10 days. Although iASPP expression may have reappeared after 7 days in the IC4 cell line I believe that the impact of iASPP knock down for up to 7 days is sufficient to observe any obvious difference in invasion pattern.

Using Cell Profiler software an invasion index was calculated which takes into account the average depth of the tumour, the number of tumours and the area of invading tumour islands (Jenei et al., 2011). Figure 5.9A shows representative images of the organotypic cultures of IC4 and IC15 cell lines depleted for iASPP and control. The invasion index for IC4 cells depleted for iASPP has no obvious differences compared to si-control (Figure 5.9B). Additionally, this is the same for IC15. However, when analysing the organotypic images, I noticed a minor difference in the size/number of colonies invading. In both cell lines depleted for iASPP the number of tumour islands invading looked to be increased but the size of these islands seemed to be smaller. Further analysis of this using the cell profiler software shows a non-significant trend towards an increase in the number of invading islands with iASPP depletion, but with the number of cells invading remaining stable (Figure 5.9C).

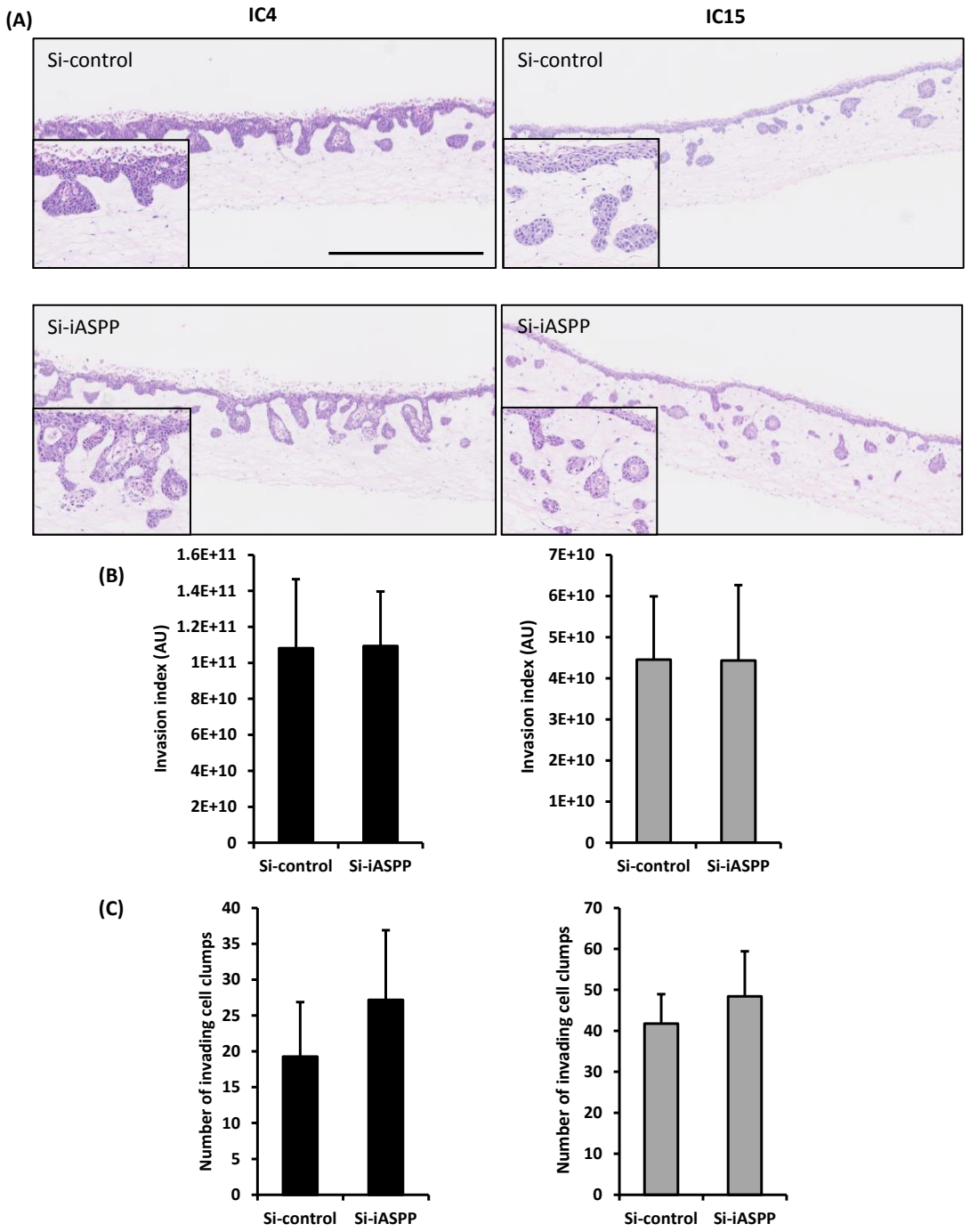


Figure. 5.9. 3D Organotypic models show cSCC silenced for iASPP affects invasion. (A) Representative images of 3D organotypic cell cultures using two cSCC cell lines, IC4 and IC15, treated with si-control or si-iASPP (si-pool). Scale bar represents 300µM. (B) Graphs display the invasion index of treated cSCC cells in 3D organotypic cell cultures. (C) Graphs display the number of invading clumps in si-control and si-iASPP (si-pool) treated cultures. Error bars represent the SEM of three independent experiments performed in duplicate.

5.2.5. MicroRNA array and qRT-PCR validation

The discovery that depletion of iASPP promotes cSCC cells to become more migratory/invasive while also gaining resistance to apoptosis was surprising due to the commonly reported role for iASPP as an oncogene. Analysis of a microRNA array performed to investigate the effects on microRNA expression when iASPP is silenced in cSCC cells provided a possible insight into why iASPP has this effect on the cell. The data from the microRNA array showed that when iASPP is silenced, miR-205-5p becomes decreased in both IC4 and IC18 cell lines but not in N-TERT (Figure 5.11A). MiR-205-5p has recently been linked with p63 and epithelial-to-mesenchymal (EMT) marker - zinc finger E-box binding homeobox 1 (ZEB1) - in bladder, prostate and breast cancer cells (Figure 5.10) (Tucci et al., 2012; Tran et al., 2012; De Cola et al., 2015). EMT is the process that enables an epithelial cell to adopt a mesenchymal cell phenotype allowing it to become more motile and invasive while also gaining a high resistance to apoptosis (Gregory et al., 2008). Expression of ZEB1 is a hallmark of EMT. ZEB1 promotes EMT by transcriptionally repressing adhesion protein E-cadherin (Sánchez-Tilló et al., 2010)

In a lethal subset of bladder cancer cells, muscle-invasive bladder cancer, ΔNp63 is highly expressed (Tran et al., 2012). Conversely, in metastatic prostate cancer p63 expression is lost (Tucci et al., 2012). Despite these obvious differences in p63 function, both authors found the same signalling pathway to be functional in both prostate and bladder cancer. Consistent with the increase in ΔNp63 expression in bladder cancer cells is the high miR-205-5p expression linked to adverse clinical outcomes (Tran et al., 2012). In support of this association, in prostate cancer when p63 expression is downregulated, miR-205-5p is lost in human metastastic prostate cancer tumour samples (Tucci et al., 2012).

The overexpression of both TAp63 and ΔNp63 in prostate cancer cell lines promoted an increase in miR-205-5p levels (Tucci et al., 2012). A chromatin Immunoprecipitation (ChIP) and luciferase reporter assay using the upstream region of miR-205-5p and subsequent transfection of ΔNp63 confirmed the ability of ΔNp63 to directly promote the miR-205-5p promoter (Tucci et al., 2012). Equally, silencing of ΔNp63α resulted in a decrease in miR-205-5p expression in bladder cancer cell lines (Tran et al., 2012). Prior to these investigations, the miR-205-5p 3'UTR had been demonstrated to directly target ZEB1 (Gregory et al., 2008). The loss of ZEB1 along with several other markers including ZEB2, Twist and Vimentin, is a hallmark change of EMT (Kalluri & Weinberg, 2009), although a decrease in all markers is not

an essential measure of EMT (Biddle et al., 2011). p63 is able to negatively regulate EMT by indirectly targeting ZEB1 through miR-205-5p (Tucci et al., 2012; Tran et al., 2012).

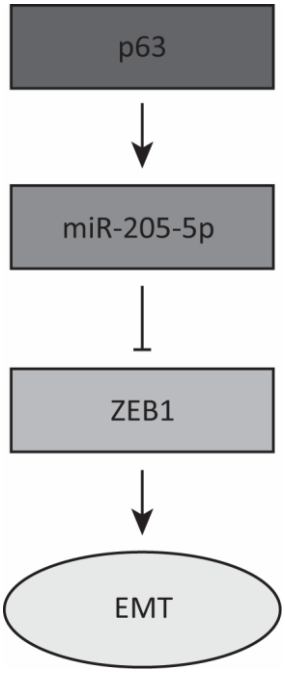


Figure 5.10. Schematic showing the relationship between p63, miR-205-5p and ZEB1 in relation to EMT. Figure adapted from Tran et al. (2012).

Taking these previous discoveries into account and in light of the previous data in this chapter showing that silencing of iASPP increases the migration/motility of cSCC cells whilst also gaining resistance to apoptosis, it was hypothesised that a decrease in iASPP and therefore p63 (based on data in chapter 4) may result in a reduction in miR-205-5p - leading to an increase in ZEB1 levels and, in turn, promotion of EMT. Also noteworthy is the recent finding that ASPP2 binds and inhibits ZEB1 in breast cancer cells. It is the N-terminus of ASPP2 (1-360 a.a.), however, that is able to repress ZEB1 through inhibiting B-catenin from transactivating ZEB1. The N-terminus of ASPP2 is different to the N-terminus of iASPP (Wang et al., 2014c).

To validate the microRNA array results TaqMan qPCR were carried out. In all three cSCC cell lines silencing of iASPP resulted in a decrease of miR-205-5p, regardless of p53 mutation

status. In the case of IC4 and IC15 this was significant (Figure 5.11B) (IC4 $p = 0.01$; IC18 $p = 0.01$). N-TERT cells depleted for iASPP did not display a change in miR-205-5p levels (Figure 5.11B).

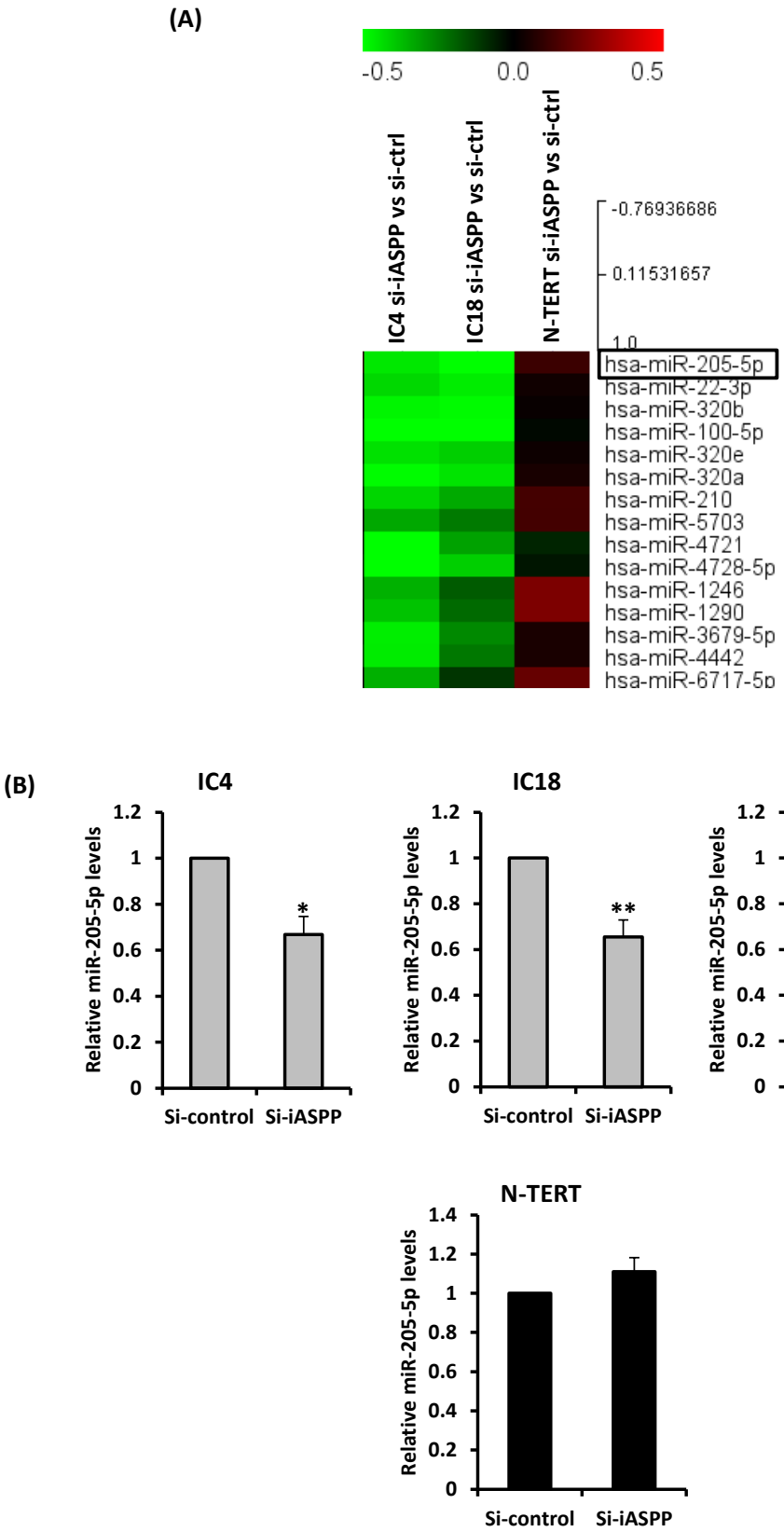

(A)
-0.5
0.0
0.5
IC4 si-iASPP vs si-ctrl
IC18 si-iASPP vs si-ctrl
N-TERT si-iASPP vs si-ctrl
-0.76936686
0.11531657
1.0
hsa-miR-205-5p
hsa-miR-22-3p
hsa-miR-320b
hsa-miR-100-5p
hsa-miR-320e
hsa-miR-320a
hsa-miR-210
hsa-miR-5703
hsa-miR-4721
hsa-miR-4728-5p
hsa-miR-1246
hsa-miR-1290
hsa-miR-3679-5p
hsa-miR-4442
hsa-miR-6717-5p
(B)
IC4
IC18
IC15
N-TERT
Relative miR-205-5p levels
Si-control
Si-iASPP
*
**

Figure 5.11. MicroRNA array and qRT-PCR validation. **(A)** Heat map displaying log-fold change values of microRNA showing the greatest decrease in expression in IC4 and IC18 cSCC cells in contrast to N-TERT cells treated with si-control versus si-iASPP (si-pool) - Agilent platform. **(B)** Cells treated with si-control or si-iASPP (si-pool) were analysed by qRT-PCR for miR-205-5p expression. MicroRNA levels were normalised to internal control microRNA – RNU48. The fold change of si-iASPP compared to si-control is shown. Error bars represent the SEM of three independent experiments performed in triplicate. Statistical analysis was performed using a two-tailed, unpaired Students t-test, comparing the si-control to si-pool. P-value ≤ 0.05 = *, ≤ 0.01 = **.

5.2.6. Depletion of miR-205-5p levels results in an increase of ZEB1

Having established in the previous section that iASPP is able to modulate miR-205-5p expression in cSCC, I wanted to investigate whether miR-205-5p was able to control ZEB1 in cSCC. Previous studies investigating the relationship between miR-205-5p and ZEB1 had been performed in malignant mesothelioma, bladder, prostate, ovarian and breast cancer (Bracken et al., 2008; Gregory et al., 2008; Tran et al., 2012; Tucci et al., 2012; Fassina et al., 2012; Wang et al., 2012; Chao et al., 2014; Lee et al., 2014a; Lee et al., 2014b; Zhang et al., 2014; De Cola et al., 2015; Niu et al., 2015). To the best of my knowledge this relationship has not been studied in cSCC.

To assess whether miR-205-5p was able to affect ZEB1 expression in cSCC, cell lines were treated with Anti-miR-205-5p, which are single stranded nucleic acids able to bind and specifically to target microRNA for inhibition. To ensure the specificity of this microRNA TaqMan qPCR assays were carried out assessing the effectiveness of Anti-miR on miR-205-5p (Figure 5.12A). In both cell lines the levels of miR-205-5p were decreased by over 90%. An increase in ZEB1 levels can be observed in both cSCC cell lines transfected with Anti-miR-205-5p, with IC18 having a significant increase (Figure 5.12B) (IC18 $p = 0.04$).

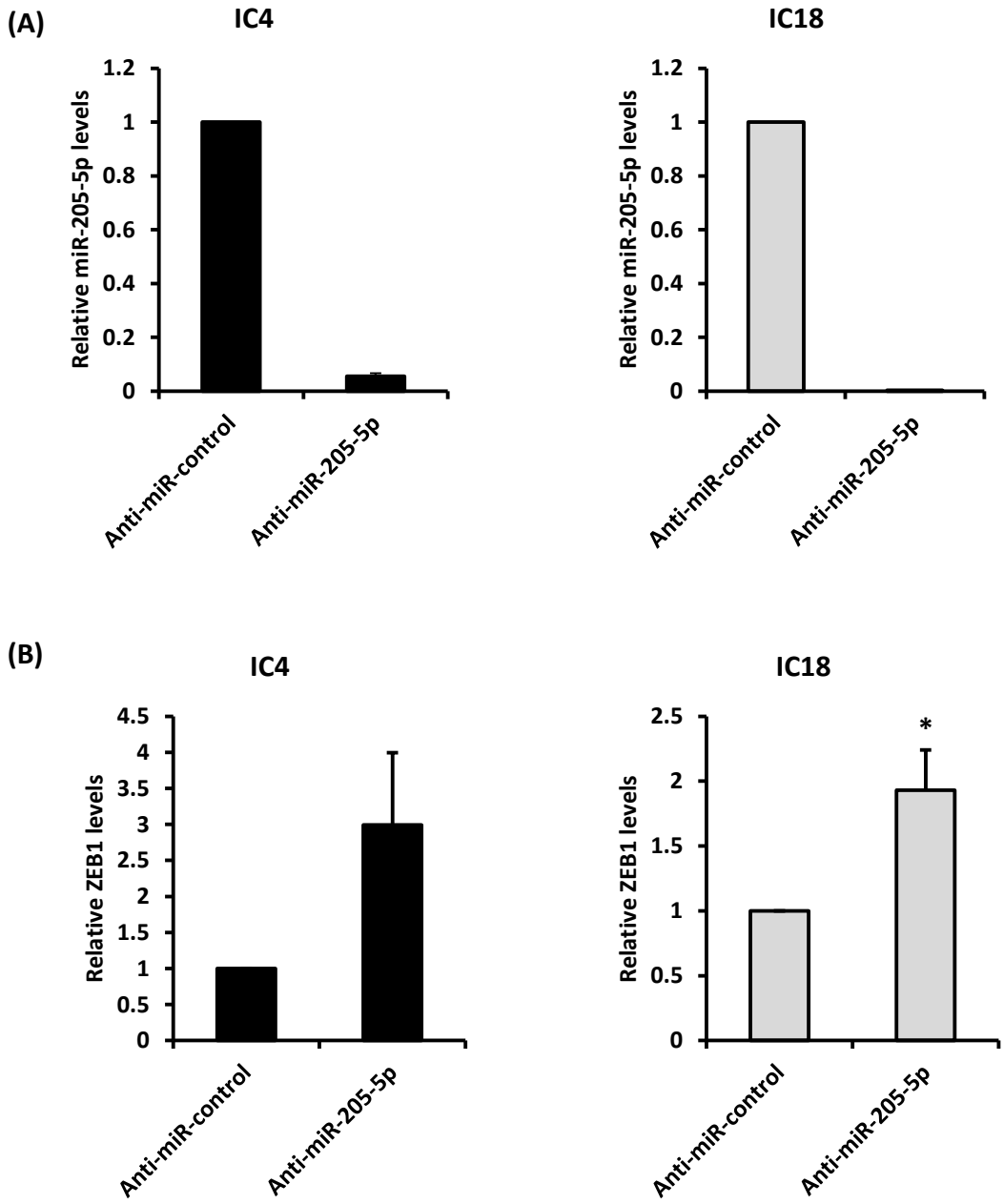


Figure 5.12. Depletion of miR-205-5p levels results in an increase of ZEB1. (A) QRT-PCR with IC4 and IC18 cells was used to validate the effectiveness of Anti-miR-205-5p treatment. Graphs show the fold change of Anti-miR treated cells compared to control. **(B)** QRT-PCR measuring the relative expression of ZEB1 in cells either treated with a non-targeting control (Anti-miR control) or treated with Anti-miR-205-5p. MicroRNA levels were normalised to internal control microRNA – RNU48. The fold change of Anti-miR control compared to Anti-miR-205-5p is shown. Error bars represent the SEM of three independent experiments performed in triplicate. Statistical analysis was performed using a two-tailed, unpaired Students t-test. P-value ≤ 0.05 = *.

5.2.7. Suppression of iASPP allows ZEB1 expression to increase

My data show that silencing iASPP causes a decrease in miR-205-5p levels and that silencing of miR-205-5p promotes an increase of ZEB1 in cSCC. To confirm whether iASPP is able to indirectly affect ZEB1 expression levels, I silenced iASPP and measured the effect on ZEB1 by qPCR. In addition to ZEB1, I also investigated the levels of other EMT markers; ZEB2, Twist-1, E-cadherin and vimentin. In human bladder cancer cells Tran et al., (2012) found that silencing ΔNp63α and miR-205-5p increased the expression of both ZEB1 and ZEB2. Tucci et al., (2012) were able to demonstrate a relationship between ΔNp63/miR-205-5p and E-cadherin. Loss of E-cadherin is associated with EMT (Kalluri & Weinberg, 2009). Overexpression of both ΔNp63 and miR-205-5p in prostate cancer cell lines increased E-cadherin expression correlating with a decrease in EMT (Tucci et al., 2012). During the same treatments another EMT marker highly expressed such as vimentin, was decreased by overexpression of ΔNp63 and miR-205. Twist-1 expression is another commonly used marker for EMT (Kalluri & Weinberg, 2009).

cSCC cell lines were silenced for iASPP and assessed by qPCR for the levels of ZEB1, ZEB2, Twist-1, E-cadherin and vimentin (Figure 5.13A). In all three cSCC cell lines analysed, ZEB1 was significantly increased upon iASPP depletion (IC18 $p = 0.0082$; IC4 $p = 0.0455$; IC15 $p = 0.0039$). No other significant trends were observed. The levels of ZEB1 were then investigated at the protein level by western blotting (Figure 5.13B). Consistent with the qPCR data, western blots showed silencing of iASPP in IC4 caused an increase in ZEB1 protein expression. This experiment has only been performed once however and is therefore still preliminary data. Future work will need to replicate and quantify this data.

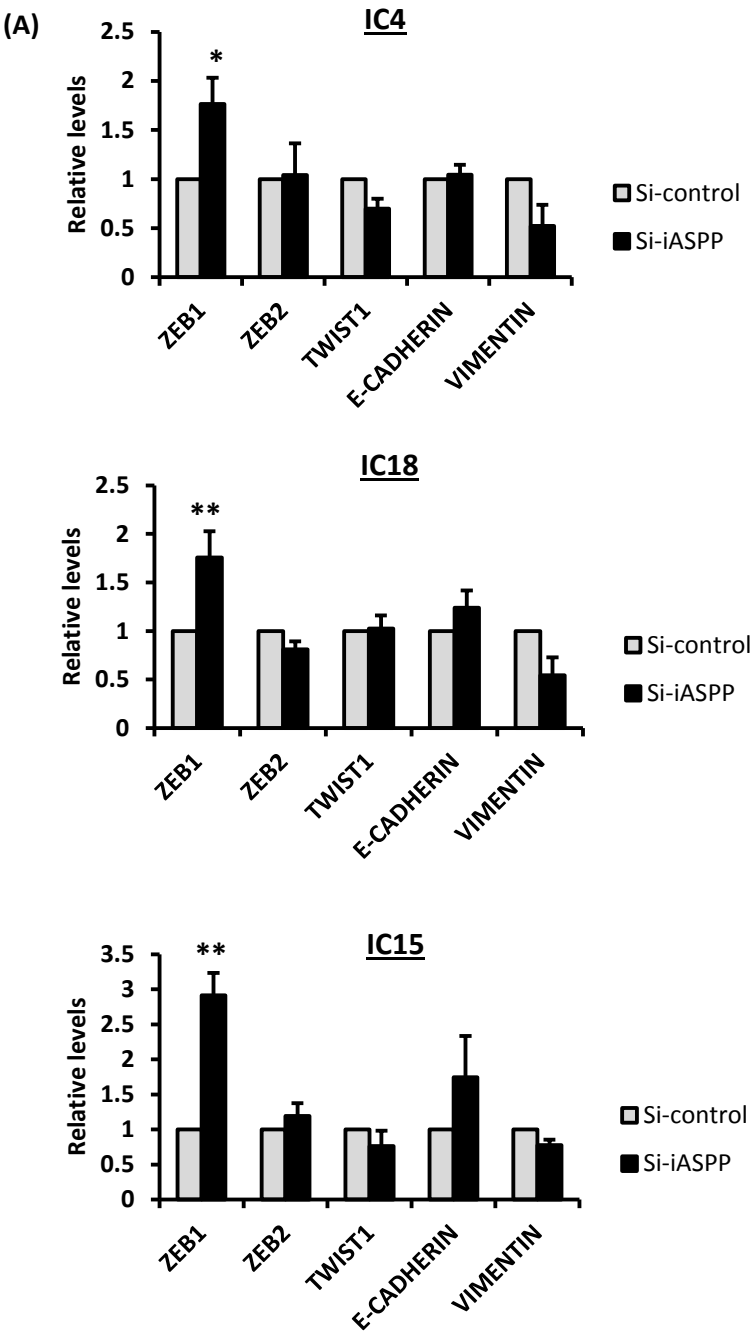
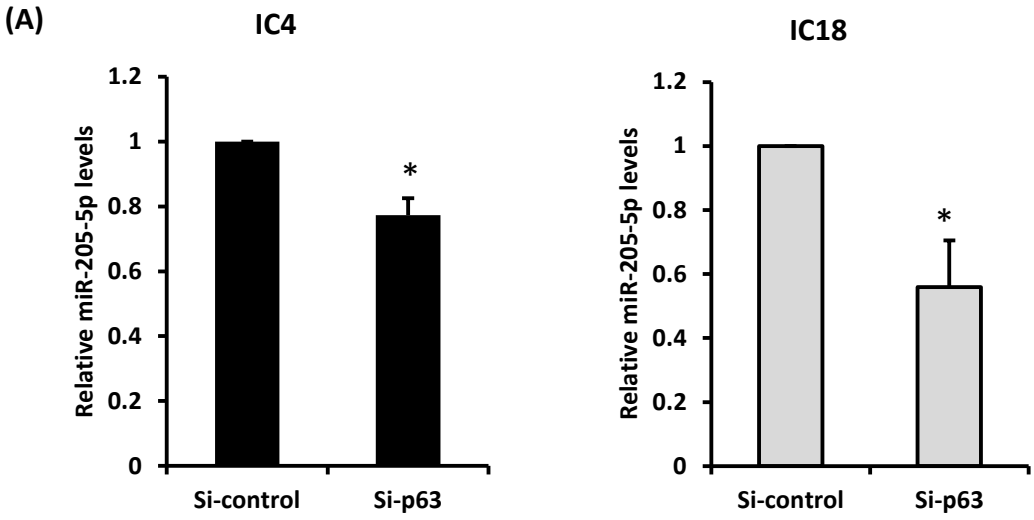
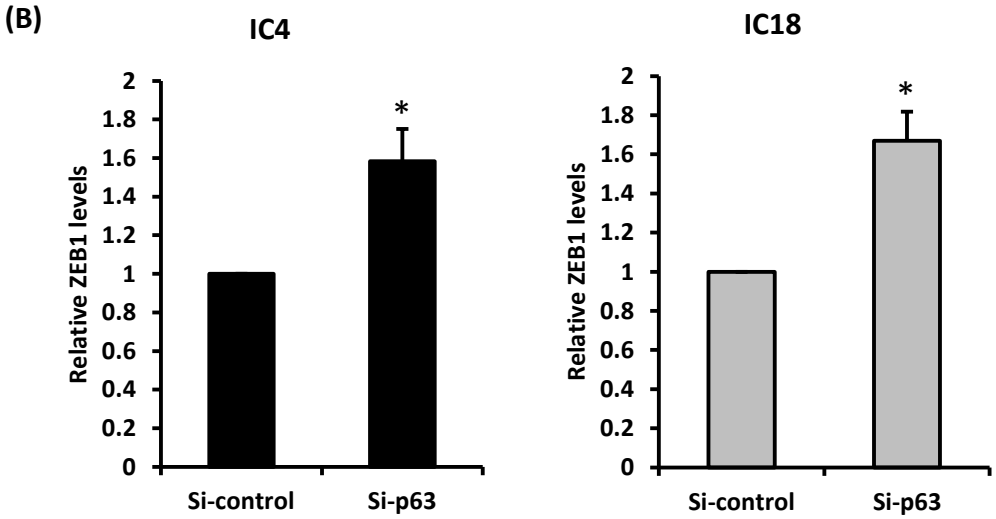

(A)
IC4
Relative levels
2.5
2
1.5
1
0.5
0
*
ZEB1
ZEB2
TWIST1
E-CADHERIN
VIMENTIN
Si-control
Si-iASPP
IC18
**
IC15
3.5
3
(B)
Si-control
Si-iASPP
ZEB1
iASPP
GAPDH
124
100
37

Figure 5.13. Effect of iASPP on markers of EMT. (A) cSCC cell lines were silenced for iASPP (si-pool) and screened for markers of EMT by qRT-PCR. Housekeeping gene GUS was used as an internal control. Error bars represent the SEM of three independent experiments performed in triplicate. Statistical analysis was performed using a two-tailed, unpaired Students t-test, comparing the si-control to si-pool P-value ≤ 0.05 = *, < 0.01 = **. **(B)** Western blot showing the effect of silencing iASPP on ZEB1 expression levels in IC4 cSCC cell line. GAPDH was used as a loading control. This Western blot was only performed once and is therefore a preliminary result.

5.2.8. Silencing p63 decreases miR-205-5p levels and increases ZEB1 expression

p63 was initially found to be the regulator of the miR-205-5p/ZEB1 axis in bladder and prostate cancer and more recently in breast cancer (Tucci et al., 2012; Tran et al., 2012; De Cola et al., 2015). To date this has not been investigated in cSCC. To investigate whether this mechanism is occurring in cSCC cells I silenced two cSCC cell lines and using TaqMan PCR analysed the effects of this silencing on miR-205-5p. In both cell lines I observed a significant decrease in miR-205-5p upon p63 depletion (Figure 5.14A) (IC4 $p = 0.0119$; IC18 $p = 0.0392$). Following on from this I investigated the effects of silencing p63 in relation to ZEB1 levels. ZEB1 levels were analysed by qPCR. Both IC4 and IC18 cell lines silenced for iASPP displayed significantly higher levels of ZEB1 compared to si-control (Figure 5.14B) (IC4 $p = 0.0260$; IC18 $p = 0.0107$). The levels of ZEB1 were then investigated at the protein level by western blotting (Figure 5.14C). Consistent with the qPCR data, western blots showed silencing of p63 in IC4 caused an increase in ZEB1 protein expression. This experiment has only been performed once however and is therefore still preliminary data. Future work will need to replicate and quantify this data.


(A)
IC4
IC18
Relative miR-205-5p levels
1.2
1
0.8
0.6
0.4
0.2
0
*
Si-control
Si-p63


(B)
IC4
IC18
Relative ZEB1 levels
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Si-control
Si-p63

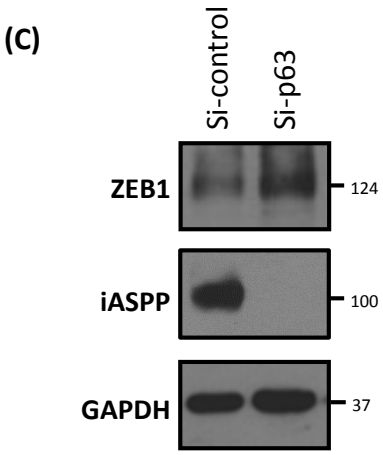

(C)
Si-control
Si-p63
ZEB1
iASPP
GAPDH
124
100
37

Figure 5.14. Effect of silencing p63 on miR-205-5p and ZEB1. (A) Cells treated with si-control or si-p63 (si-pool) were analysed by qRT-PCR for miR-205-5p expression. MicroRNA levels were normalised to internal control microRNA – RNU48. The fold change of si-iASPP compared to si-control is shown. **(B)** cSCC cell lines were silenced for p63 and screened for ZEB1 by qRT-PCR. Housekeeping gene GUS was used as an internal control. Error bars represent the SEM of three independent experiments performed in triplicate. Statistical analysis was performed using a two-tailed, unpaired Students t-test, comparing the si-control to si-pool. P-value ≤ 0.05 = *. **(C)** Western blot showing the effect of silencing p63 on ZEB1 expression levels in IC4 cSCC cell line. GAPDH was used as a loading control. This Western blot was only performed once and is therefore a preliminary result.

5.3. Summary

In order to understand why iASPP is so highly expressed in cSCC and thus what role iASPP plays in cancer, I wanted to investigate the pathophysiological effects of iASPP in cSCC. The effect of iASPP on cell proliferation previously been well documented in both normal skin and various types of cancer (Pang et al., 2010; Lu et al., 2010; Zhang et al., 2011; Lin et al., 2011; Chen et al., 2011, Li et al., 2011; Chikh et al., 2011; Notari et al., 2012; Lu et al., 2013; Zhao et al., 2013; Chen et al., 2014b; Liu et al., 2014; Morris et al., 2014; Pandolfi et al., 2015; Wang et al., 2015a). Confirming these data in cSCC, I found that silencing iASPP greatly affects the ability of cSCC cells to proliferate. Further investigation into this observation found that the role of iASPP in proliferation was linked to cyclin D2 expression. Upon silencing of iASPP, the expression of cyclin D2, a cell cycle protein required to complex with CDK4/6 enabling the cell to cycle through G1/S phase, was decreased. These data supported previous findings in HaCaT cells and transferred them to a tumour setting (Chikh et al., 2011). The effect of iASPP on cell proliferation was also examined using colony forming assay. In all three cSCC cell lines, cells depleted for iASPP show a reduction in the number of colonies formed, supporting the previous data.

iASPP has been well established as an inhibitor of apoptosis in cancer cells in both a p53 dependent- and p53-independent manner (Bergamaschi et al., 2003; Cai et al., 2012b). However, although a well-documented inhibitor of apoptosis in a cancer setting, conflicting studies provide evidence that iASPP does not always play an anti-apoptotic role (Laska et al. 2007; Pinto et al., 2010; Chikh et al., 2014). Studies reported a pro-apoptotic function of iASPP in both non-transformed cells and precancerous lesions but also more recently in cancerous cells (Laska et al., 2007; Pinto et al., 2010; Kramer et al., 2015). Chikh et al., (2014) also demonstrated another novel function of iASPP in the skin as an autophagy inhibitor. To explore the role of iASPP in relation to apoptosis in cSCC, apoptosis was induced in three cSCC cell lines using chemotherapeutic drugs, etoposide and cisplatin. To assess the effects of iASPP on apoptosis an Annexin-V-FITC assay was used and cells were analysed by flow cytometry. As the majority of the literature describes the apoptotic role of iASPP as p53-dependent, cell lines with both wild type and mutated p53 were included to investigate any differences. cSCC cells silenced for iASPP and either untreated or treated with etoposide or cisplatin did not appear to have an increase in apoptosis. The percentage of cells undergoing apoptosis remained the same in si-iASPP treated cells compared to si-control treated cells. To investigate whether iASPP was involved in inhibiting autophagy instead of apoptosis, both

N-TERT and cSCC cell lines treated with si-control and si-iASPP were run on a western blot and probed with an LC3 antibody. Consistent with Chikh et al. (2014), N-TERT cells silenced for iASPP displayed higher levels of LC3-II, a marker of autophagy, compared to control. Interestingly, however, none of the cSCC cells depleted for iASPP showed higher levels of LC3-II lipidation providing evidence that in cSCC iASPP may not be an inhibitor of autophagy. In all three cell lines LC3-II expression was high compared to N-TERT. This apparent high level of autophagy could explain the resistance of si-iASPP cells to apoptosis. A study into cSCC found an increased resistance of advanced stage cSCC cells to cisplatin treatment and high levels of autophagy, measured by high accumulation of LC3-II (Claerhout et al., 2010). It would be interesting to test autophagy inhibitor, 3-methyladenine, on these cells and see if an increase in apoptosis was observed (Claerhout et al., 2010).

Further research into other physiological effects of iASPP in a cancer setting has been very limited. At present, to the best of my knowledge only a few observational studies have reported correlations between iASPP expression and invasive tumour tissue/metastasis (Liu et al., 2010; Cao et al., 2013; Kim et al., 2015b). iASPP deficient mice develop ARVC known to be caused by a defect in desmosome integrity. Additionally, desmosomal complexes in HaCaT cells silenced for iASPP become dysregulated. It is interesting how loss of iASPP expression causes defects in desmosomal integrity, a marker of cell invasion and metastasis, but iASPP is predominantly regarded as an oncogene. To investigate this, cSCC cell lines were depleted of iASPP and their motility tracked using Metamorph Image Analysis Software. Still images from these experiments show cells treated with si-control adhere to each other forming tight cell colonies. In contrast, cells depleted for iASPP appear to form less cell-cell contacts and therefore fewer colonies. Additionally, cells silenced for iASPP had a higher mean velocity than cells treated with si-control. This apparent loss of cell-cell contact appears to cause the cell to become more motile. Investigating this further using transwell inserts measuring the effect of iASPP on cell migration showed that cSCC cells silenced for iASPP were able to migrate more than non-silenced cells.

Investigating further the effects of increased cell motility and migration upon depletion of iASPP, 3D organotypic cultures were produced. Using Cell Profiler software, an invasion index, which takes into account the average depth of the tumour, the number of tumours and the area of invading tumour islands, was calculated (Jenei et al., 2011). The invasion index, however, showed no obvious differences between si-iASPP and si-control treated

cells. The only slight difference observed was the difference in the size/number of colonies invading. In both cell lines depleted for iASPP the number of tumour islands invading looked to be increased but the size of these islands seemed to be smaller. iASPP depletion induces a slight increase in the number of invading islands although the number of cells invading is still the same.

The finding that iASPP had a role in inhibiting invasion/migration of cSCC cells whilst not playing an anti-apoptotic role was surprising in view of the reported role for iASPP as an oncogene. These features of iASPP observed in cSCC cell lines were consistent with the hallmarks of EMT. To investigate this further a microRNA array was performed with cSCC cells and N-TERT silenced for iASPP and non-targeting siRNA. Within this array one of the top microRNA downregulated upon iASPP depletion in cSCC but not N-TERT was the miR-205-5p. This microRNA had previously been demonstrated to interact and regulate ZEB1 expression, a hallmark of EMT. Additionally, a few papers had demonstrated the ability of p63 to control the miR-205-5p and in turn ZEB1, inhibiting EMT. In chapter 4 I have shown that iASPP is able to regulate p63 expression via miR-211-5p and that in turn p63 is able to directly regulate iASPP expression. Considering these data, I sought to discover whether iASPP was involved in this scheme of events to inhibit EMT. Firstly, I validated the microRNA array data showing that silencing of iASPP resulted in a decrease of miR-205-5p. I then proceeded to show that, in cSCC cells, miR-205-5p was still able to control ZEB1 expression. To prove that iASPP was linked to this pathway I silenced iASPP and checked the effect on ZEB1 levels in addition to other markers of EMT. QPCR and western data confirmed that iASPP was able to affect ZEB1 expression. The involvement of p63 in this pathway in cSCC cell lines was confirmed by observing a decrease in miR-205-5p levels upon p63 silencing and an increase in ZEB1. Taken together these experiments imply that iASPP is an EMT inhibitor in cSCC.

Chapter 6: Discussion and future work

6.1. Nuclear p63 and cytoplasmic iASPP are highly expressed in cSCC

6.1.1. p63 and iASPP are highly expressed in cSCC in vitro

Non-melanoma skin cancer has the highest incidence of any cancer in the UK. Both cSCC and BCC are classified as non-melanoma skin cancer. Although BCC is more prevalent than cSCC, cSCC presents greater morbidity and has a higher incidence of metastasis than BCC (Madan et al., 2010). The metastatic potential of cSCC is still low compared to melanoma however the morbidity on the patient and the cost of cSCC on the healthcare system is high (Eedy, 2000; Guy et al., 2015). UV is the most important risk factor of cSCC, however, the direct molecular mechanism that causes cSCC is unclear. Several genes have been implicated including p53 and Notch1, both of which are tumour suppressor genes commonly mutated in cSCC (Brash et al., 1991; Ziegler et al., 1994; Forbes et al., 2010; South et al., 2012; Samarasinghe & Madan, 2012). Additionally, high expression/mutation of oncogenes EGFR, Ras, c-myc and p63 has also been documented (Pelisson et al., 1996; Senoo et al., 2001; Reis-Filho et al., 2002; Wrone et al., 2004; Boukamp, 2005; Dotto and Glusac, 2006; Kolev et al., 2008; Alomari et al., 2014).

p63 contains two major isoforms; ΔNp63 and TAp63. These isoforms can be further divided into splice variants; α, β, γ, δ and ε (Yang et al., 1998; Mangiulli et al., 2009). ΔNp63 is a well-established oncogene that is able to repress TAp63, p73, full length p53, p53 isoform Δ133p53, and is highly expressed in many different cancers (Yang et al., 1998; Liefer et al., 2000; Ratovitski et al., 2001; Rocco et al., 2006; Marcel et al., 2012). Expression of p63 is used as a marker for epithelial cancers such as cSCC and a handful of reports have demonstrated high p63 expression in cSCC (Senoo et al., 2001; Reis-Filho et al., 2002; Wrone et al., 2004; Dotto and Glusac, 2006; Alomari et al., 2014). TAp63 is documented as a tumour suppressor gene. However, recently in melanoma, TAp63 was shown to behave as an oncogene (Matin et al., 2013). In this study an antibody was used that detects all the isoforms of p63. In all 10 of the cSCC cell lines analysed only ΔNp63α was highly expressed, supporting previous published data. When compared to N-TERT cells, used as a control for 'normal keratinocytes,' p63 was highly expressed in all cSCC. Each cSCC cell line used in this study had undergone a targeted gene screen and thus the mutational status of p53, Notch1 and Ras was known. p53 is a tumour suppressor gene and belongs to the same family as p63. Typically, p53 is mutated

in 60% of cSCC and this is reflected in the panel of cSCC cell lines used for this project. Several studies have investigated the effect of mutant p53 on p63. Mutant p53 is able to interact with TAp63 via its DBD leading to a decrease in the tumour suppressive transcriptional activities of TAp63 (Li and Prives 2007). Conversely, high levels of mutant p53 and upregulated expression of ΔNp63 are often reported suggesting that mutant p53 may not have the same effect on ΔNp63 as it does TAp63. p53 has a lower binding affinity for ΔNp63 than TAp63 (Gaiddon et al., 2001). Conflicting evidence shows that hot-spot R175H mutant p53 actually induces the expression of ΔNp63 after DNA damage (Lanza et al., 2006).

All but one of the cSCC cell lines used in this project were obtained from a typical sun exposed area, for example the scalp, the back of hand or ear. The IC15 cell line, however, although classified as an cSCC, arises from the penis and is HPV-16 positive. Thus, despite the wild type status of p53 in this cell line, in this instance, p53 is silenced by HPV oncoprotein E6 and is therefore non-functional. Notch1, a validated tumour suppressor gene in cSCC, is also mutated in approximately 60% of cSCC cases (Forbes et al., 2010; South et al., 2012). In our panel of cSCC cell lines Notch1 is mutated in 7/10 cell lines. This is unsurprising when coupled with the finding that ΔNp63 is upregulated in cSCC. Wild type Notch1 is able to suppress p63 levels in keratinocytes, however, high p63 expression inhibits Notch1 activity (Nguyen et al., 2006; Restivo et al., 2011). Ras, a classic oncogene, is mutated in 10-20% of cSCC tumours and interacts with ΔNp63 in the skin to promote proliferation of squamous cell carcinoma in mice (Keyes et al., 2011). Despite these interactions, when comparing both p63 protein and RNA expression to the mutational profile of the cell lines there appeared to be no strong correlation. There were also no correlations with clinicopathologic features such as immunocompetence status, age, site of tumour and differentiation status of tumour.

iASPP is an inhibitor of p53 and has previously been considered as a potential therapeutic target for tumour p53 re-activation. iASPP is highly expressed in many tumours and our group has recently shown that it signals with p63 in a feedback loop in the skin (Chikh et al., 2011). Structural studies showed that iASPP preferentially binds to p63 over p53 and additionally, binds p63 with a higher affinity than ASPP family member ASPP2, a validated interactor with p63 (Robinson et al., 2008). The expression levels of iASPP have been investigated in many cancers but, to date, not in cSCC. Recent studies have, however, demonstrated the importance of iASPP in HNSCC and cervical SCC (Liu et al., 2012; Cao et al., 2013). In this study using 10 cSCC cell lines and N-TERT control I found an upregulation of

iASPP at both the protein and RNA level. As iASPP is already highly expressed in the normal skin there was only a slight increase in cSCC. There appeared to be no clear correlation between mutated p53 and iASPP levels. This was expected as data suggest iASPP works upstream from full-length p53 and does not signal through mutant p53 (Bergamaschi et al., 2003; Bergamaschi et al., 2006). Additionally, no clear correlation between Notch1 and iASPP was observed. In the skin, p63 signals upstream of Notch1 and iASPP signals upstream of p63. Thus, high levels of iASPP may have indirectly signalled low levels of Notch1 (Nguyen et al., 2006; Restivo et al., 2011). iASPP is an inhibitor of differentiation in the normal skin. I therefore examined iASPP expression compared to the differentiation status of the cell lines, hypothesising that iASPP may be higher in the poorly differentiated tumours. In cSCC cell lines this proved not to be the case. However, the *in vitro* system of cSCC cell lines was probably unable to represent the alternative differentiation statuses of the tumours, as identified using involucrin, a marker of keratinocyte differentiation (Watt, 1983).

6.1.2. Nuclear p63 and cytoplasmic iASPP are highly expressed in cSCC, both in vitro *and* in vivo

In order to translate these findings into an *in vivo* setting tumours were stained for both iASPP and p63. In total 106 tumours were obtained from patients with differing clinicopathological features. The sample selection collected mirrored SCC in the general population as 73% of the tumours were from men and the majority of samples were from sun exposed sites with 39% on head and neck and 36% upper limb. In 92/106 (87%) tumours there was perilesional epidermis present allowing an internal control. Sections were scored for the percentage of cells stained multiplied by the intensity of the staining producing a scoring index. An antibody against all p63 isoforms was used. At the time of staining there was limited availability of specific ΔNp63α antibodies suitable for staining. Based on the prominent expression of ΔNp63α in all the cSCC cell lines, however, I could assume that it was ΔNp63α that was positively stained. As mentioned previously, strong p63 expression is a marker for epithelial cancers and in particular cSCC. p63 is used as a marker due to its high expression in epithelial cells. For this reason it is also highly expressed in the normal skin. Therefore, no further increase in p63 staining could be observed in the tumours as all perilesional epidermal and cSCC samples scored positively for p63. A couple of reports had shown high p63 expression in poorly differentiated SCC (Kargi et al., 2007; Alomari et al., 2014). In this case p63 was highly expressed regardless of differentiation status. Additionally,

in melanoma cells cytoplasmic p63 was expressed; however, in cSCC tumours p63 protein expression was limited to the nucleus (Matin et al., 2013).

iASPP is located in both the nucleus and cytoplasm. When initially discovered as RAI (Rel A-Associated Inhibitor), iASPP was a nuclear protein (Yang et al., 1999; Slee et al., 2004) The later discovery of the full-length version of iASPP found the N-terminus responsible for its cytoplasmic location and the C-terminus, the one found in RAI, responsible for its nuclear setting (Bergamaschi et al., 2003; Slee et al., 2004). As the C-terminus and thus nuclear section of iASPP is responsible for binding to its binding partners such as p53, reports into iASPP in other SCC tumours finding that cytoplasmic iASPP expression in the tumour correlated with worse patient prognosis seem confusing. In HNSCC cytoplasmic iASPP was an independent prognostic factor (Liu et al., 2012). However, a strong case for nuclear iASPP was put forward by Lu et al. (2013). In melanoma, nuclear iASPP was highly expressed and phosphorylated via cyclin B/CDK1. Phosphorylated iASPP could be visualised on a western blot as a second slower migrating band. Treatment using JNJ-7706621, an inhibitor of CDK1 and thus phosphorylation of nuclear iASPP, was able to reduce tumour size and growth in mouse melanoma models (Lu et al., 2013). Data published after this discovery by Morris et al. (2014) also primarily focussed on the importance of nuclear iASPP in prostate cancer and researchers were able to view this double iASPP band on western blot, denoting cytoplasmic iASPP and phosphorylated nuclear iASPP. Closer inspection of this paper, however, also showed the importance for cytoplasmic iASPP in prognosis.

6.1.3. High cytoplasmic and low nuclear iASPP expression may be associated with a more aggressive tumour

Due to these conflicting findings, iASPP was scored for the intensity and percentage of cells stained with nuclear iASPP and cytoplasmic iASPP separately. The data showed that the normal epidermis expressed high levels of nuclear iASPP and lower levels of cytoplasmic iASPP, as previously shown (Chikh et al., 2011, Notari et al., 2011). Interestingly, when comparing the normal epidermis to the tumours, there appeared to be a switch in staining with the levels of cytoplasmic iASPP rising and the levels of nuclear iASPP decreasing. This suggests that cytoplasmic iASPP may be more important for tumourigenesis of cSCC than nuclear iASPP. To investigate this further, collaborators from The Barts Cancer Institute performed a multivariate analysis of nuclear versus cytoplasmic iASPP staining compared with the clinicopathologic features of the patients. Although these data were not quite

significant, a trend was observed regarding the differentiation status of the tumour and location of iASPP. Not only is there a switch in staining from normal epidermis to tumour, but the intensity of this switch is dependent on the differentiation status of the tumour. The poorly differentiated and thus more aggressive tumours display much higher levels of cytoplasmic iASPP and are nearly all negative for nuclear iASPP. Due to the smaller numbers of poorly differentiated cSCC tumours, the power of the data were limited. However, the trend relating to differentiation is very striking and supports previous papers correlating cytoplasmic iASPP to worse prognosis (Jiang et al., 2011; Liu et al., 2012; Cao et al., 2013; Morris et al., 2014). Thus depletion of nuclear iASPP/increase of cytoplasmic iASPP may be a marker for aggressive cSCC.

To investigate this phenomenon further, I fractionated cell lines into nuclear and cytoplasmic fractions and analysed the distribution of protein expression of iASPP. Fractionation of the cSCC cell lines confirmed the high cytoplasmic/low nuclear expression distribution observed in the tumours. I also stained cSCC cell lines for iASPP. To try to produce an *in vitro* model of the normal epidermis containing higher nuclear iASPP levels compared to cSCC and to see what effect the differentiation status had upon the location of iASPP, I stained N-TERT cells alongside a sample of cSCC cells of distinct differentiation stage. No correlation could be seen between iASPP location and differentiation in the cell lines. However, this was likely due to their loss of differentiation phenotype in culture as mentioned earlier. Furthermore, N-TERT cells showed predominantly cytoplasmic iASPP expression and therefore were not representative of the normal epidermis. This was potentially due to the artificial 2D environment that cells grow in.

To investigate the cell lines further I attempted to recreate the *in vivo* differentiation status of the tumours *in vitro*. By adding calcium to cell lines I was able to induce a degree of differentiation (Pillai et al., 1988). This differentiation was confirmed by the upregulation of involucrin, a marker of keratinocyte differentiation (Watt, 1983). In support of what was observed *in vivo* I found that cells which were well differentiated expressed higher levels of nuclear iASPP. Cells grown in low calcium conditions and thus not as well differentiated, had higher cytoplasmic iASPP expression and lower nuclear expression, thus mimicking the tumour data. Although these *in vitro* data correlate with my tumour data, Chikh et al. (2011) found that in normal keratinocytes treated for an increasing period of time with calcium total iASPP expression actually decreased. As Chikh et al. (2011) did not separate the nuclear and

cytoplasmic fractions and as the nuclear fraction of iASPP is so low, the increase of nuclear iASPP would not have been evident in the total iASPP section. Thus these data do not contradict their finding. What is interesting is the role for iASPP in differentiation in normal keratinocytes, iASPP is an inhibitor of differentiation in the normal skin. iASPP is expressed in the nucleus of proliferating basal cells and becomes more cytoplasmic in well differentiated cells (Notari et al., 2011). This contradicts the findings of my tumour data and perhaps shows the differences between the role of iASPP in the tumour versus the non-tumourigenic cells. Thus, I have identified an important difference in iASPP location when comparing normal skin to the tumour that could be used as a marker for cSCC.

To investigate the issue of phosphorylated nuclear iASPP I used the cell lysis protocol from Lu et al. (2013). This is different to the lysis protocol I had been using previously. I wanted to determine whether phosphorylated iASPP could be detected in my cSCC cell lines and if so why nuclear iASPP did not appear to be the potential oncogenic target in cSCC that it was in melanoma. Using a melanoma cell line as a control I was able to observe this second upper phosphorylated band in the melanoma cell line but not in the cSCC or N-TERT cell line, confirming that the presence of phosphorylated iASPP may be cell type specific. This was further confirmed by Lu et al. (2013) who found no phosphorylated iASPP in HaCaT cells, an immortalised keratinocyte cell line. In the study on melanoma, high nuclear iASPP expression correlated with wild type p53 expression. The tumours expressing wild type p53 had high levels of nuclear phosphorylated iASPP along with high MDM2 and cyclin B1 expression (Lu et al., 2013). This observation provides further evidence that the importance of phosphorylated iASPP may be tumour specific as p53 is mutated in a high proportion of cSCC tumours. It would be interesting for future work to test other SCC cell lines for the presence of phosphorylated iASPP and determine why iASPP is not phosphorylated in SCC. It could be speculated that the reason I saw a decrease in nuclear iASPP expression in the poorly differentiated tumours was due to the inability of the antibody to detect phosphorylated iASPP. Lu et al. (2013), however, used the same antibody to detect phosphorylated iASPP as I do in this thesis.

It is important to discover why the C-terminal nuclear fraction of iASPP that is responsible for gene binding is downregulated in cSCC. Why does full length iASPP with both nuclear and cytoplasmic sections locate to particular areas? iASPP does not have a nuclear localisation signal. However, a recent publication confirmed that iASPP was able to enter the nucleus via

an importin-independent mechanism (Lu et al., 2014). Slee et al. (2004) suggested that cytoplasmic iASPP may reside in the cytoplasm to prevent ASPP1 and ASPP2 (also predominantly cytoplasmic) binding to p53. p53 is expressed in the cytoplasm and thus the regulation of p53 via iASPP may also occur extranuclear and independent of transactivation. A study found that p53 lacking a DBD and thus lacking its transactivation function was still able to trigger apoptosis (Haupt et al., 1995; Chipuk et al., 2004). Cytoplasmic p53 is able to induce apoptosis via mitochondrial outer membrane permeabilization (MOMP), triggering the release of pro-apoptotic factors, cytoplasmic p53 also acts as an inhibitor of autophagy (Green and Kroemer 2009). Furthermore, studies found p53 was able to induce apoptosis in cells lacking a nucleus (Haupt et al., 1995 Chipuk et al., 2004).

To further investigate this line of research the following experiments could be performed:

1. Collect additional poorly differentiated samples. Staining of these samples with iASPP may provide statistical significance correlating high cytoplasmic/low nuclear iASPP expression with a poorly differentiated/more aggressive tumour.

2. Generate constructs encoding different regions of iASPP representative to nuclear and cytoplasmic segments. Transfect iASPP plasmids into cSCC cell lines/mouse models and look for effects on tumour size and growth. Does cytoplasmic iASPP have a greater effect on the aggressiveness of the tumour/metastatic profile?

3. Confirm the cell type specificity of iASPP phosphorylation by investigating whether iASPP is phosphorylated by CDK1 in cSCC. Screen the cell lines for cyclin B1 expression and compare with melanoma cell lines. Test JNJ-7706621, a CDK1 inhibitor, on cSCC cell lines/mice exhibiting cSCC tumours to assess the importance of phosphorylated iASPP in cSCC.

6.2. p63 and iASPP are controlled by an autoregulatory feedback loop via miR-211-5p in cSCC

6.2.1. iASPP and p63 are controlled by an autoregulatory feedback loop in cSCC

In the normal skin p63 and iASPP colocalise in the basal epidermis where they both exist in the nucleus. The data obtained from the first chapter of this thesis, showing that in cSCC

tumours iASPP becomes less nuclear and more cytoplasmic, was therefore initially surprising. A handful of papers have now demonstrated the ability of p63 and iASPP to interact (Robinson et al., 2008; Chikh et al 2011; Notari et al., 2011; Cai et al., 2012b). In the normal skin p63 is able to directly interact with iASPP by binding to the iASPP promoter affecting its expression. In HaCaT and N-TERT cells silencing of p63 decreases the expression levels of iASPP and silencing iASPP decreases p63 (both TA and ΔN) protein expression (Chikh et al., 2011). Thus it was interesting to examine whether the p63-iASPP feedback loop reported in the normal skin was dysregulated in cSCC due to this change in iASPP location. In SCC, high levels of p63 correlate with low levels of ASPP2 expression (Tordella et al., 2013). Upon transfection of ASPP2 into cells, 70% of cells lost p63 expression. Further analysis found that cytoplasmic ASPP2 represses nuclear p63 expression via binding and counteracting IκB, enabling nuclear expression of RelA/p65 NF-κB to repress p63 expression (Tordella et al., 2013).

In order for genes to interact with each other they must be in the same location. To investigate the effects of p63 on iASPP I silenced p63 (all isoforms) in a subset of cSCC cell lines. Three cell lines were chosen for their differing levels of iASPP and p63 expression and also for their mutational status as determined by a targeted gene screen. Despite these differences and the apparent difference in iASPP location, silenced p63 was still able to reduce iASPP expression at both the mRNA and protein level. To investigate this further I used subcellular fractionation to separate the nuclear and cytoplasmic fraction in all three of the cell lines. Subcellular fractionation carried out in the previous chapter, found both cytoplasmic and nuclear iASPP present but cytoplasmic iASPP present to a much higher extent. When p63 was silenced in this instance it was observed that the expression of both nuclear and cytoplasmic iASPP was decreased. This was interesting as I had predicted that only nuclear iASPP would be reduced due to p63 transcriptionally regulating iASPP in the nucleus. One explanation for this could be that iASPP is targeted by p63 in the nucleus and then shuttled out into the cytoplasm. Although iASPP does not contain a nuclear localisation signal, it is able to enter the nucleus by an importin-independent mechanism via tha RaDAR (RanGDP/Ankyrin repeat) pathway (Lu et al., 2014). The Ankyrin repeats present in the C-terminus of iASPP, specifically two adjacent 13th hydrophobic residues, harbour a code enabling RanGDP to bind and complex with nuclear transport factor 2 resulting in the import of the complex into the nucleus (Lu et al., 2014). Research into how iASPP exits the nucleus could shed further light on the feedback loop system.

As I had confirmed that p63 was able to affect the expression of iASPP in the cell I wanted to investigate whether iASPP affected p63 protein expression. In the study by Chikh et al. (2011), silencing of iASPP was able to affect p63 at the protein level but not the mRNA level. Additionally, Robinson et al. (2008) proved that iASPP binds to p63 directly regulating its activity. In chapter 4 of this thesis, my data show that silencing of iASPP affects p63 at the protein level only hinting at another level of control between iASPP and p63. This was independent of p53.

6.2.2.MicroRNA control the iASPP/p63 autoregulatory feedback loop in cSCC

A lot of data have been published showing the importance of microRNA in regulating genes. p63 is a good example of this and is the target of many microRNA. Chikh et al. (2011) showed that iASPP was able to regulate p63 in the normal skin via two microRNA, miR-574-3p and miR-720. Based on the previous evidence I wanted to identify whether miR-574-3p and miR-720 were still controlled by iASPP and able to regulate p63 in cSCC. Whilst investigating this, it became apparent that miR-720 was no longer classified as a microRNA but a tRNA (Schopman et al., 2010). For the purpose of this thesis it was therefore excluded from study.

To analyse the effects of iASPP on miR-574-3p, I silenced iASPP and measured the levels of miR-574-3p. In all three cSCC cell lines the levels of miR-574-3p did not increase suggesting that iASPP was no longer controlling miR-574-3p in cSCC. These data were later confirmed by a microRNA array showing that levels of miR-574-3p were not affected upon silencing of iASPP. Surprisingly, this was also the case in N-TERT cells leading us to speculate that silencing differences may be responsible - stable knockdown versus transient silencing. Despite these differences however the effect of miR-574-3p on p63 was still evident by overexpressing miR-574-3p and witnessing a decrease in ΔNp63α protein expression. This, coupled with the finding that *in vivo* miR-574-3p expression levels are very low in cSCC tumour tissue compared to the normal skin, could suggest a novel potential therapeutic approach for decreasing but not eliminating ΔNp63α expression in cSCC by overexpressing miR-574-3p.

As mentioned above, during this project I carried out a microRNA array to attempt to identify in cSCC a cell specific microRNA whose expression would be affected by silencing of iASPP and which could be an alternative to the microRNA reported by Chikh et al. (2011) in normal

keratinocytes depleted for iASPP. When analysing the data I focussed on microRNA that became upregulated upon iASPP silencing. Further to this using microRNA.org, miRDB.org and miRWalk 2.0, I identified microRNA that were predicted to target p63. Two microRNA; miR-328-3p and miR-211-5p, were upregulated upon iASPP silencing and predicted to target p63. Due to the ability of microRNA to target and regulate many different genes, microRNA frequently have conflicting roles in different cancers. This is evident with both the miR-328-3p and miR-211-5p. MiR-328-3p is oncogenic in brain metastasis in NSCLC and glioma invasion. On the other hand, several studies have found tumour suppressive properties of miR-328-3p in glioblastoma, osteosarcoma, colorectal and breast cancer (Wu et al., 2012; Xu et al., 2012; Yang et al., 2014; Wang et al., 2015b; Yaun et al., 2015). MiR-211-5p has been found to promote tumourigenesis in oral, colorectal and HNSCC but suppresses tumourigenesis in melanoma, pancreatic and sebaceous carcinomas (Chang et al., 2008; Mazar et al., 2010; Levy et al., 2010; Sakurai et al., 2011; Boyle et al., 2011; Cai et al., 2012a; Giovannetti et al., 2012; Xu et al., 2012; Chu et al., 2013; Maftouh et al., 2014; Sümbül et al., 2015; Tetzlaff et al., 2015).

In order to test whether both the miR-328-3p and the miR-211-5p were able to actually target p63, pre-miR mimics of each microRNA were transfected into the cells and the expression of p63 was analysed. Interestingly, transfection of miR-211-5p caused a decrease in ΔNp63α expression suggesting miR-211-5p was able to regulate p63. Over expression of miR-328-3p on the other hand, did not have an effect on p63 expression. This could be for a number of different reasons. Firstly, although target prediction programmes are a very useful starting point for discovering microRNA targets they are not context specific and do not take into account the levels of expression of both the microRNA and the gene in a particular cell. Moreover target prediction programmes do not consider how the expression of other genes that are targets of the same microRNA affect the binding of the microRNA to the predicted target (Barbato et al., 2009).

Validation of the microRNA array by qPCR also highlighted the observation that this phenomenon was not occurring in N-TERT cells suggesting a tumour specific pathway. Additionally of note, is the difference in microRNA profile in the IC15 cell line. The IC15 cell line is an HPV-16 positive cell line and is therefore different to classic UV induced SCC. Despite the inability of iASPP to control miR-211-5p expression in IC15 cells, miR-211-5p was still able to target p63 and downregulate the expression in IC15 cells strengthening the evidence that

miR-211-5p may be a more universal target of p63. Transfection of HEK293 cells with a p63 plasmid containing a luciferase reporter gene and Pre-miR-211-5p showed a reduction in luciferase activity of over 30% compared to transfection of mutant p63, i.e. one that miR-211-5p is unable to bind to. HEK293 cells were used as a model due to their lack of detectable endogenous p63 expression. Transfecting a p63 plasmid into an already highly expressing p63 cSCC cell line may have weakened the result. Thus, this is the first report of the miR-211-5p controlling p63 and could be used as a therapeutic target to help regulate oncogenic p63 levels in cSCC. Once p63 had been confirmed as a direct novel target for miR-211-5p, the potential therapeutic effects of miR-211-5p were tested in an *in vitro* setting. Interestingly overexpression of miR-211-5p in cSCC was able to reduce the proliferation of cancer cells and limit the cSCC cells ability to form cell colonies. This finding supports data showing that iASPP is a regulator of proliferation in cSCC cells (Chapter 5) and highlights miR-211-5p as a potential therapeutic target able to regulate the downstream effects of iASPP without directly targeting iASPP itself.

To investigate this line of research further, the following experiments could be performed:

- A chromatin immunoprecipitation with iASPP and miR-211-5p to demonstrate whether iASPP is able to directly bind and target miR-211-5p.

- Confirm the absence of effect of miR-211-5p in N-TERT cells in primary keratinocyte cell lines and perform *in situ* hybridisation staining of miR-211-5p in cSCC tumour sections compared to normal skin to help clarify if miR-211-5p could be a suitable therapeutic target/biomarker for cSCC.

- Further characterise the epigenetic switch between normal skin and cSCC. Use an alternative silencing system, for example, an inducible lentiviral system or CRISPR to confirm that the miR-574-3p is no longer controlling iASPP in cSCC.

6.3. iASPP is essential for the proliferation of cSCC but is an inhibitor of EMT

6.3.1. iASPP regulates the proliferation of cSCC cells in vitro

iASPP is usually known as an oncogene due to its overexpression in several cancers and its ability to inhibit the apoptosis-inducing gene – p53 (Bergamaschi et al., 2003; Bergamaschi et al., 2006). Further to this, several studies have demonstrated that iASPP is essential for cell proliferation. Silencing iASPP and observing a decrease in cell proliferation has been observed many times, however, recently it was shown that, at least in the normal skin, iASPP was affecting cell proliferation via its ability to regulate cyclin D2, a gene essential for cell cycle progression past G1/S phase. Li et al. (2010) and Chikh et al. (2011) have confirmed the effect of iASPP on cyclin D proteins in glioblastoma and HaCaT cells, respectively. In support of these findings in a subset of cSCC cells, cell line growth was slowed when iASPP was depleted from the cell and protein analysis showed a decrease in cyclin D2 expression. Additionally, colony assays intended to measure the ability of a cell to grow into a colony were restricted when iASPP was silenced.

The fact that ΔNp63 also regulates the proliferation of a cell and that iASPP and p63 are regulated via an autoregulatory feedback loop suggests that the proliferation of a cell is controlled not solely by iASPP or p63 but by the autoregulatory feedback loop as a whole (Sbisà et al., 2006). Further work is required in this area to investigate the mechanism of how iASPP is able to affect cyclin expression and whether downregulation of p63 also affects cyclin expression in this way. These data show the oncogenic potential of iASPP (and ΔNp63) in cSCC and the importance is highlighted by the finding that miR-211-5p is able to inhibit these effects downstream.

6.3.2. Silencing of iASPP in cSCC cell lines does not increase apoptosis

The ability of iASPP to inhibit apoptosis both dependent of p53 and independently of p53, via p63 and p73 instead, is well documented (Bergamaschi et al., 2003; Cai et al., 2012b). For this thesis I measured the percentage of cSCC cells undergoing apoptosis when silenced for iASPP compared to si-control. Cells were treated with the chemotherapeutic drugs etoposide, cisplatin and also in no-drug conditions. I hypothesised I would see an increase in apoptosis. However, although I observed an expected increase in apoptosis in cells treated with drugs compared to no drugs, I did not see a difference in percentage of apoptotic cells between cells expressing iASPP and cells depleted for iASPP. In contrast to the well-known

role of iASPP as an inhibitor of apoptosis, iASPP was first discovered as a p65/rel A binding protein (Yang et al., 1999). P65/Rel A is a subunit of the NFκB complex that is involved in the immune and inflammatory responses of the cell and can inhibit apoptosis. Although only investigated in non-transformed cells, Laska et al. (2007) demonstrated that cells silenced for iASPP and treated with etoposide had a reduction in apoptosis. Treatment of these cells with an NFκB inhibitor reversed this effect.

In a cancer that is frequently mutated for p53, iASPP can induce apoptosis independently of p53, via p63 and p73. Interestingly iASPP is able to carry out apoptosis via p63 when it is tumour suppressive (Cai et al., 2012b). In cSCC however, p63 is predominantly oncogenic. Moreover, a reduction in iASPP expression reduces cell proliferation. Cells which proliferate at a slower rate are more resistant to apoptosis. Slowly proliferating cells are more resistant to chemotherapy than rapidly proliferating cells (Sultana et al., 2003). Furthermore, in the skin, iASPP has already been shown to be an inhibitor of autophagy and not apoptosis. Autophagy is the conserved process of intracellular degradation that occurs within a cell via fusion of a double-membraned organelle, known as an autophagosome, with a lysosome/vacuole (Xie and Klionsky 2007). Chikh et al., (2014) found that treatment of keratinocytes with both UVB and anti-cancer treatment staurosporine, cells silenced for iASPP exhibited a greater resistance to apoptosis. Keratinocytes depleted of iASPP had decreased levels of pro-apoptotic gene NOXA and increased levels of lipidated LC3, a marker of autophagy (Naik et al., 2007; Chikh et al., 2014). In contrast, in cSCC, I found no effect on LC3 lipidation when iASPP was silenced compared to control cells. These data were supported by the inclusion of N-TERT cells in which an increase in LC3 lipidation was observed upon iASPP depletion, highlighting the potential difference between normal and cancerous cells. High levels of LC3-II were observed in all cSCC cell lines. A previous study found that increased resistance of advanced stage cSCC cells to cisplatin treatment was, in part, due to high levels of autophagy (Claerhout et al., 2010). It could be speculated therefore that my panel of cSCC cells were resistant to apoptosis due to high LC3-II levels. Recent work described a controversial pro-apoptotic role for iASPP in melanoma. The data suggested that in melanoma iASPP could promote apoptosis via the acetylation and thus stabilization of p53 and TAp73, through acetyltransferases p300 and CBP (Kramer et al., 2015). These data conflict with other results showing an apoptotic role in melanoma; nevertheless, it would be interesting in the future to investigate this further in cSCC.

6.3.3. cSCC cells silenced for iASPP display an EMT phenotype

The result from the apoptosis assay was unexpected given the oncogenic activity of iASPP with regard to proliferation. However, this can be linked to the ability of iASPP to potentially inhibit EMT, of which resistance to apoptosis is a key hallmark (Lamouille et al., 2014). The first hints that iASPP may be involved in EMT came from the observation that when iASPP is depleted, cells become more motile and lose their cell-cell contacts. Support in the literature for these findings showed that iASPP was essential for cell-cell contact (Chikh et al. 2011; Notari et al., 2015). EMT is the process whereby epithelial cells transform into mesenchymal cells. During this process the epithelial cells lose their cell adhesion properties and become more migratory and invasive. Thus the process of EMT is commonly found in metastasising tumours (Lamouille et al., 2014). As well as increased motility in cells depleted for iASPP, cells were also able to migrate faster when depleted for iASPP. In correlation with these findings, loss of ΔNp63α in SCC cell lines promoted cell migration and increased cell motility (Barbieri et al., 2006). These data provide evidence that it is not iASPP alone but the autoregulatory feedback loop as a whole that is able to control cell migration and motility.

Organotypic cultures generated with iASPP silenced cells provided clues that the invasion pattern of cSCC cells may change upon iASPP depletion. Although the number of cells invading was similar, the pattern of invasion was different. In 3D models, iASPP silencing produced smaller invading islands compared with the control ones. When invading tumour islands start to break up and become smaller they ultimately become more invasive and lead to metastasis. Conflicting evidence has correlated iASPP expression and invasive tumour tissue/metastasis (Liu et al., 2010; Cao et al., 2013; Kim et al., 2015b). However these studies were limited to observational findings.

6.3.4. MiR-205-5p is downregulated in cSCC cells depleted for iASPP – releasing the control of EMT related gene – ZEB1

During the analysis of the microRNA array performed in chapter 4, I had noticed a significant decrease in miR-205-5p in cSCC cells silenced for iASPP but not in N-TERT. MiR-205-5p is well known in the p63 field as several studies have shown p63 binds to and regulates miR-205-5p (Tran et al., 2012; Tucci et al., 2012). The finding in this thesis that iASPP and p63 are able to regulate each other via a feedback loop in cSCC made the effect of iASPP on miR-205-5p an interesting area in which to carry out further study. MiR-205-5p is an upstream inhibitor of

EMT, binding to and regulating EMT marker ZEB1. p63 has been shown to inhibit EMT; however, a contradictory report showed that ΔNp63 was able to promote EMT via TGFβ in keratinocytes (Oh et al., 2011). Furthermore, a recent study also linked ASPP2 and ZEB1 in breast cancer cell lines where ASPP2 expression was capable of inducing MET, the reverse process of EMT. Overexpression of ASPP2 caused a decrease in ZEB1 expression (Wang et al., 2014c). Thus I wanted to investigate the effects of both iASPP and p63 on miR-205-5p and ZEB1.

QPCR validation experiments confirmed the effect of iASPP on miR-205-5p. Moreover, qPCR experiments performed when p63 was silenced in cSCC support the previous literature showing that silencing p63 has a negative effect on miR-205-5p levels. p53 is an inhibitor of EMT and miR-205-5p has a p53RE (Chang et al., 2011). However, like Tran et al. (2012), I was able to rule out p53 from the effect of p63 on miR-205-5p due to the presence of a p53 mutation in the IC18 cell line. TAp63 is able to inhibit metastasis due to its ability to regulate Dicer, a component of the microRNA processing system (Su et al., 2012). Although a pan-p63 siRNA is used, my data mainly focus on ΔNp63. Due to the low/undetectable expression of TAp63 in cSCC cells, inhibition of TAp63 would not have been appreciated.

QPCR experiments found that in cSCC cells, depletion of miR-205-5p causes an increase in ZEB1. To confirm whether iASPP and p63 were signalling upstream of miR-205-5p and promoting the downstream effects of ZEB1, both p63 and iASPP were silenced and ZEB1 levels analysed. Upon silencing of both iASPP and p63, an increase in ZEB1 was observed. Although silencing of iASPP was able to increase ZEB1 expression, no effects were detected on the expression of the other EMT markers analysed. For EMT, however, it is not necessary for all EMT markers to change (Biddle et al., 2011). These findings confirm that iASPP and p63 signal upstream of miR-205-5p in order to mitigate its effects and are the first report of this kind in cSCC. These data provide a new function for iASPP in cSCC that has not been reported before.

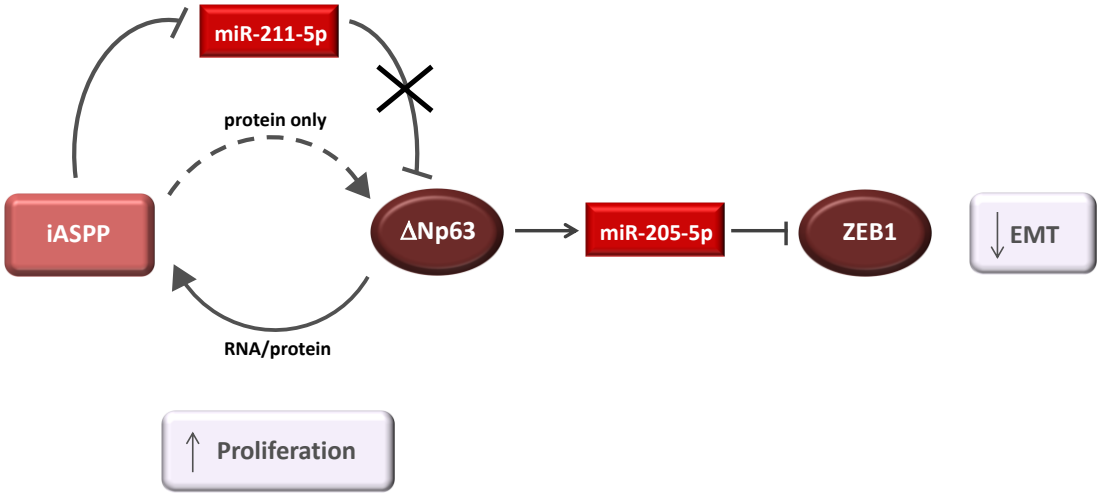


Figure 6.1. iASPP/p63 autoregulatory feedback loop in cSCC. High expression levels of both iASPP and p63 maintain the feedback loop in cSCC and increase proliferation in the cells. Low levels of miR-211-5p in the tumour prevent the inhibitory effects of the microRNA on p63. High iASPP and p63 contribute towards high miR-205-5p expression, inhibiting EMT marker ZEB1 causing a decline in EMT.

In chapter 3 of this thesis I described high cytoplasmic iASPP/low nuclear iASPP as a potential marker for poorly differentiated cSCC. Poorly differentiated cSCC is the most aggressive form of cSCC and is characterised by the break-up of large tumour islands into smaller invading islands. In these more aggressive invading cells, nuclear iASPP expression is lost, yet there is high cytoplasmic expression. In chapter 5 I have shown that iASPP is essential for proliferation and, consistent with this, cytoplasmic iASPP is highly expressed in these aggressive tumours. Contradictory evidence in chapter 5, however, describes iASPP as a potential inhibitor of EMT - a process more likely to occur in a more aggressive tumour. This raises some difficult questions relating to why the apparent tumourigenic cytoplasmic iASPP, highly expressed in aggressive tumours, would be also inhibiting EMT in these tumours. Conversely it could be speculated that it is nuclear iASPP mainly contributing to the EMT phenotype in cSCC and this may therefore explain the loss of nuclear iASPP in the aggressive tumours thus allowing EMT to take place. Given that cytoplasmic iASPP is the predominant form expressed in the cSCC cell lines - where a role for iASPP as an inhibitor of EMT has been developed however - needs further explaining.

Taken together these experiments imply that iASPP may be inhibiting EMT in cSCC. This seems paradoxical given the apparent oncogenic status of iASPP in cancer and the role of EMT in tumour metastasis. EMT however is a dynamic process and is only required for cells escaping the primary tumour. When researching the role of ΔNp63α in bladder cancer, Tran et al. (2012) came up against a similar issue. ΔNp63α is highly oncogenic yet seems to inhibit EMT in bladder cancer. Tran et al. (2012) however speculated that circulating tumour cells may express lower ΔNp63α than cells in the primary and metastatic tumour. iASPP may also follow the same pattern. In support of this I have shown that iASPP is essential for proliferation. Cells in transit do not proliferate. However, proliferation is essential for cells of a primary tumour or cells that have subsequently undergone mesenchymal-to-epithelial transition at a distant site (Tran et al., 2012; Tsai et al., 2012). At present there is a lot of attention on inhibiting EMT to prevent metastasis. Tsia et al. (2012), however, argue that therapeutically blocking EMT in SCC could be disadvantageous. After primary tumour removal there is often a delayed onset of metastasis. Tumour cells that undergo EMT and migrate to other sites of the body sometimes remain dormant in these sites. Delayed onset metastasis occurs when these dormant cells undergo reverse EMT (mesenchymal-to-epithelial transition, MET) allowing the cells to proliferate and form metastases. Inhibiting MET may provide a better treatment angle by preventing dormant tumour cells from forming metastases (Tsia et al., 2012).

To investigate this line of research further, the following experiments could be performed:

- Determine whether iASPP has a direct effect on miR-205-5p or whether this is via p63:
    - Insert the miR-205 promoter region in a luciferase reporter gene and measure luciferase activity in the presence iASPP
    - Perform a ChIP experiment to assess whether iASPP binds to miR-205-5p directly.
- Investigate whether nuclear and cytoplasmic iASPP have differing functions with regard to proliferation and EMT.

6.4. Clinical relevance

The finding that iASPP/p63 may exhibit a proliferative, oncogenic phenotype in cSCC coupled with an ability to inhibit EMT raises questions regarding the potential for iASPP/p63 as a

therapeutic in cSCC. By targeting iASPP/p63 we may slow down the growth of the tumour but also promote the cells to undergo EMT. Additionally, depleting iASPP/p63 from cSCC cells would need to be targeted to tumour cells, perhaps as topical therapy, limiting contact with normal skin as iASPP/p63 is essential for cell homeostasis. In melanoma, a small-molecule inhibitor of cyclin B/CDK1, JNJ-7706621 that prevents the phosphorylation and nuclear localisation of iASPP has been shown to be effective in suppressing the growth of melanoma cells (Lu et al., 2013). In cSCC we have failed to show a phosphorylated version of iASPP in addition to showing a tumourigenic phenotype for nuclear iASPP over cytoplasmic iASPP. Using JNJ-7706621 may therefore only be relevant in specific cell types.

Targeting iASPP in tumours with wildtype p53 is a popular concept in the literature. A34 (a small peptide derived from p53 linker) binds directly to iASPP and inhibits the iASPP-p53 interaction (Qiu et al., 2015). In cSCC, however, where a high percentage of tumours actually express mutant p53, this may prove ineffective. Recent studies have found a handful of upstream targets of iASPP including miR-124 and the Hedgehog pathway (Zhao et al., 2013; Chen et al., 2014a; Liu et al., 2014; Pandolfi et al., 2015). Targeting these upstream molecules has been shown, however, to induce the activity of wildtype p53, which is not as relevant to cSCC. Additionally, depleting iASPP will still pose the same issues of EMT and the fact that iASPP is essential for the normal skin.

Regardless of the therapeutic approaches for iASPP, loss of nuclear iASPP expression and increase of cytoplasmic expression in cSCC tumours could predict a worse outcome, however further work would need to be performed to prove this. Additionally, these data support the use of p63 expression in detecting cSCC tumours but do not support differences in p63 expression relating to differentiation status of the tumour.

MicroRNA are increasingly being considered for therapy against a variety of diseases including diabetes, inflammation, neurological disorders and, of particular relevance, cancer (Broderick and Zamore, 2011). Due to this interest, biotechnology companies are focusing on microRNA therapeutics. Even though research into microRNA is still at a relatively early stage, microRNA therapeutics are already entering clinical trials. Currently, a lot of research into cancer therapies focuses on targeting single genes. In many cases this only provides a limited response. The advantage to targeting microRNA is that they control several genes/pathways, eliciting a stronger response. This can, however, be a disadvantage as the

broad range of genes that one microRNA may target may include several oncogenes but also tumour suppressor genes in different cells/tissues. MicroRNA can be exploited in the clinic in two ways. Generating mimics to overcome a loss of function or antagonists to inhibit oncogenic microRNA (Bader and Lammers, 2011). The advantage of using mimics over antagonists is that they can be introduced into the cells using the same systems used for therapeutic siRNA (Bader and Lammers, 2011). Additionally, by expressing microRNA mimics there should be fewer off target effects in normal tissue that have high levels of these microRNA. One of the first microRNA mimics that may be used in a clinical setting is the miR-34a. The company behind the drug, miRNA Therapeutics Inc, entered the drug MRX34 into phase I clinical trials in 2013 assessing the effects of the drug on primary liver cancer, SCLC, melanoma, lymphoma, ALL, CLL and multiple myeloma (www.clinicaltrials.gov).

For this thesis, mimics of miR-574-3p/miR-211-5p or miR-205-5p could prove useful in treating cSCC. An advantage of treating cSCC is the capacity for local delivery, i.e., a topical treatment, increasing the bioavailability in diseased tissue and limiting off target effects on healthy tissue (Broderick and Zamore, 2011). By introducing these microRNA into tumourigenic cells we can increase the levels of these microRNA expressed in diseased cells enabling them to become less tumourigenic. For example, the expression of miR-574-3p in cSCC tissue by *in-situ* hybridisation staining was greatly decreased compared to the normal skin. Overexpressing miR-574-3p and miR-211-5p would allow the levels of p63 to be controlled but not depleted, as p63 is essential for the normal skin. Additionally overexpressing miR-211 and miR-574-3p is a way of inhibiting the oncogenic downstream effects of iASPP, for example cell proliferation without depleting iASPP, a gene essential for cell homeostasis in the stratified epithelia.

Treatment using mimics against the miR-547-3p and miR-211-5p could prevent the oncogenicity of iASPP and p63. However, the findings from this thesis demonstrated that downstream of iASPP and p63 is the miR-205-5p functioning as an inhibitor of EMT. By treating cells with miR-211-5p/miR-574-3p we are decreasing the levels of p63 and, in turn, potentially decreasing the levels of miR-205-5p - increasing EMT. Further work would need to be done on the downstream effects of using miR-547-3p and miR-211-5p mimics. Alternatively, combination therapies combining miR-547-3p/miR-211-5p with miR-205-5p may block the proliferation of the cells in addition to preventing EMT.

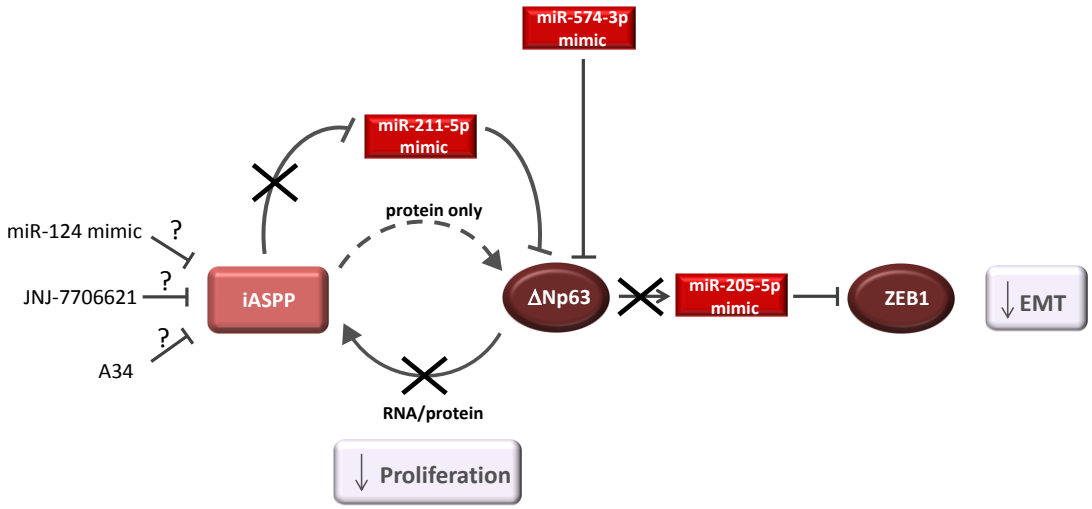


Figure 6.2. Clinical relevance of iASPP/p63 feedback loop in cSCC. Inhibitors/regulators of iASPP; JNJ, A34 and miR-124 could potentially regulate iASPP levels in cSCC decreasing the proliferation rate of the tumour. Mimics against miR-211-5p and miR-574-3p decrease p63 expression. Combination treatment with these mimics and miR-205-5p mimic may prevent EMT stimulation.

6.5. Concluding remarks

This thesis has focussed on investigations into the role of iASPP in cSCC and has sought to offer further understanding of the mechanisms occurring in cSCC. It is interesting that as in the normal epidermis, iASPP and p63 are still controlled by an autoregulatory feedback loop in cSCC – however from this thesis it is now known that in cSCC two additional microRNA are involved – miR-211-5p and miR-205-5p. Whilst the miR-211-5p has been identified as a novel regulator of p63, miR-205-5p was identified as being controlled by both iASPP and p63 and in turn affecting the EMT of the cell. In addition, insight into the difference in the location of iASPP in the cell has been provided – with a more cytoplasmic and less nuclear expression pattern occurring in the more poorly differentiated tumours. Taken together, the data presented here may provide new avenues for exploration in cSCC and potential novel therapeutic targets.

Chapter 7: References

ALDABAGH, B., ANGELES, J. G., CARDONES, A. R. & ARRON, S. T. 2013. Cutaneous squamous cell carcinoma and human papillomavirus: is there an association? *Dermatol Surg,* 39**,** 1-23.

ALOMARI, A. K., GLUSAC, E. J. & MCNIFF, J. M. 2014. p40 is a more specific marker than p63 for cutaneous poorly differentiated squamous cell carcinoma. *J Cutan Pathol,* 41**,** 839-45.

APPLIED-BIOSYSTEMS. Endogenous Controls for Real-Time Quantitation of miRNA using Taqman MicroRNA assays. Application Note.

ARORA, S., RANADE, A. R., TRAN, N. L., NASSER, S., SRIDHAR, S., KORN, R. L., ROSS, J. T., DHRUV, H., FOSS, K. M., SIBENALLER, Z., RYKEN, T., GOTWAY, M. B., KIM, S. & WEISS, G. J. 2011. MicroRNA-328 is associated with (non-small) cell lung cancer (NSCLC) brain metastasis and mediates NSCLC migration. *Int J Cancer,* 129**,** 2621-31.

ASHTON, K. J., WEINSTEIN, S. R., MAGUIRE, D. J. & GRIFFITHS, L. R. 2003. Chromosomal aberrations in squamous cell carcinoma and solar keratoses revealed by comparative genomic hybridization. *Arch Dermatol,* 139**,** 876-82.

AYLON, Y., OFIR-ROSENFELD, Y., YABUTA, N., LAPI, E., NOJIMA, H., LU, X. & OREN, M. 2010. The Lats2 tumor suppressor augments p53-mediated apoptosis by promoting the nuclear proapoptotic function of ASPP1. *Genes & Development,* 24**,** 2420-2429.

BACHELOR, M. A. & BOWDEN, G. T. 2004. UVA-mediated activation of signaling pathways involved in skin tumor promotion and progression. *Semin Cancer Biol,* 14**,** 131-8.

BADER, A, G. & LAMMERS, P, L. 2011. The Therapeutic Potential of microRNAs. *Innov Pharm Technol,* 52-55.

BARBATO, C., ARISI, I., FRIZZO, M. E., BRANDI, R., DA SACCO, L. & MASOTTI, A. 2009. Computational challenges in miRNA target predictions: to be or not to be a true target? *J Biomed Biotechnol,* 2009**,** 803069.

BARBIERI, C. E., TANG, L. J., BROWN, K. A. & PIETENPOL, J. A. 2006. Loss of p63 leads to increased cell migration and up-regulation of genes involved in invasion and metastasis. *Cancer Res,* 66**,** 7589-97.

BELL, H. S. & RYAN, K. M. 2008. iASPP inhibition: Increased options in targeting the p53 family for cancer therapy. *Cancer Res,* 68**,** 4959-4962.

BERGAMASCHI, D., SAMUELS, Y., JIN, B., DURAISINGHAM, S., CROOK, T. & LU, X. 2004. ASPP1 and ASPP2: common activators of p53 family members. *Mol Cell Biol,* 24**,** 1341-50.

BERGAMASCHI, D., SAMUELS, Y., O'NEIL, N. J., TRIGIANTE, G., CROOK, T., HSIEH, J. K., O'CONNOR, D. J., ZHONG, S., CAMPARGUE, I., TOMLINSON, M. L., KUWABARA, P. E. & LU, X. 2003. iASPP oncoprotein is a key inhibitor of p53 conserved from worm to human. *Nat Genet,* 33**,** 162-7.

BERGAMASCHI, D., SAMUELS, Y., SULLIVAN, A., ZVELEBIL, M., BREYSSENS, H., BISSO, A., DEL SAL, G., SYED, N., SMITH, P., GASCO, M., CROOK, T. & LU, X. 2006. iASPP preferentially binds p53 proline-rich region and modulates apoptotic function of codon 72-polymorphic p53. *Nat Genet,* 38**,** 1133-41.

BERGHOLZ, J. & XIAO, Z. X. 2012. Role of p63 in Development, Tumorigenesis and Cancer Progression. *Cancer Microenviron,* 5**,** 311-22.

BIDDLE, A., LIANG, X., GAMMON, L., FAZIL, B., HARPER, L. J., EMICH, H., COSTEA, D. E. & MACKENZIE, I. C. 2011. Cancer stem cells in squamous cell carcinoma switch between two distinct phenotypes that are preferentially migratory or proliferative. *Cancer Res,* 71**,** 5317-26.

BLANDINO, G. & MOLL, U. M. 2009. p63 regulation by microRNAs. *Cell Cycle,* 8**,** 1466-7.

BOLDRUP, L., COATES, P. J., WAHLGREN, M., LAURELL, G. & NYLANDER, K. 2012. Subsite-based alterations in miR-21, miR-125b, and miR-203 in squamous cell carcinoma of the oral cavity and correlation to important target proteins. *J Carcinog,* 11**,** 18.

BOUKAMP, P. 2005. Non-melanoma skin cancer: what drives tumor development and progression? *Carcinogenesis,* 26**,** 1657-67.

BOYLE, G. M., WOODS, S. L., BONAZZI, V. F., STARK, M. S., HACKER, E., AOUDE, L. G., DUTTON-REGESTER, K., COOK, A. L., STURM, R. A. & HAYWARD, N. K. 2011. Melanoma cell invasiveness is regulated by miR-211 suppression of the BRN2 transcription factor. *Pigment Cell Melanoma Res,* 24**,** 525-37.

BRACKEN, C. P., GREGORY, P. A., KOLESNIKOFF, N., BERT, A. G., WANG, J., SHANNON, M. F. & GOODALL, G. J. 2008. A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition. *Cancer Res,* 68**,** 7846-54.

BRANTSCH, K. D., MEISNER, C., SCHONFISCH, B., TRILLING, B., WEHNER-CAROLI, J., ROCKEN, M. & BREUNINGER, H. 2008. Analysis of risk factors determining prognosis of cutaneous squamous-cell carcinoma: a prospective study. *Lancet Oncol,* 9**,** 713-20.

BRASH, D. E., RUDOLPH, J. A., SIMON, J. A., LIN, A., MCKENNA, G. J., BADEN, H. P., HALPERIN, A. J. & PONTEN, J. 1991. A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proc Natl Acad Sci U S A,* 88**,** 10124-8.

BREUNINGER, H., BLACK, B. & RASSNER, G. 1990. Microstaging of squamous cell carcinomas. *Am J Clin Pathol,* 94**,** 624-7.

BRODERICK, J. A. & ZAMORE, P. D. 2011. MicroRNA therapeutics. *Gene Ther,* 18**,** 1104-10.

BROWN, V. L., HARWOOD, C. A., CROOK, T., CRONIN, J. G., KELSELL, D. P. & PROBY, C. M. 2004. p16INK4a and p14ARF tumor suppressor genes are commonly inactivated in cutaneous squamous cell carcinoma. *J Invest Dermatol,* 122**,** 1284-92.

BRUEGGER, C., KEMPF, W., SPOERRI, I., ARNOLD, A. W., ITIN, P. H. & BURGER, B. 2013. MicroRNA expression differs in cutaneous squamous cell carcinomas and healthy skin of immunocompetent individuals. *Exp Dermatol,* 22**,** 426-8.

BUSBY-EARLE, R. M., STEEL, C. M., WILLIAMS, A. R., COHEN, B. & BIRD, C. C. 1994. p53 mutations in cervical carcinogenesis--low frequency and lack of correlation with human papillomavirus status. *Br J Cancer,* 69**,** 732-7.

BYRNE, C., TAINSKY, M. & FUCHS, E. 1994. Programming gene expression in developing epidermis. *Development,* 120**,** 2369-83.

CAI, C., ASHKTORAB, H., PANG, X., ZHAO, Y., SHA, W., LIU, Y. & GU, X. 2012a. MicroRNA-211 expression promotes colorectal cancer cell growth in vitro and in vivo by targeting tumor suppressor CHD5. *PLoS One,* 7**,** e29750.

CAI, Y., QIU, S., GAO, X., GU, S. Z. & LIU, Z. J. 2012b. iASPP inhibits p53-independent apoptosis by inhibiting transcriptional activity of p63/p73 on promoters of proapoptotic genes. *Apoptosis,* 17**,** 777-83.

CANDI, E., CIPOLLONE, R., RIVETTI DI VAL CERVO, P., GONFLONI, S., MELINO, G. & KNIGHT, R. 2008. p63 in epithelial development. *Cell Mol Life Sci,* 65**,** 3126-33.

CANDI, E., RUFINI, A., TERRINONI, A., DINSDALE, D., RANALLI, M., PARADISI, A., DE LAURENZI, V., SPAGNOLI, L. G., CATANI, M. V., RAMADAN, S., KNIGHT, R. A. & MELINO, G. 2006. Differential roles of p63 isoforms in epidermal development: selective genetic complementation in p63 null mice. *Cell Death Differ,* 13**,** 1037-47.

CAO, L., HUANG, Q., HE, J., LU, J. & XIONG, Y. 2013. Elevated expression of iASPP correlates with poor prognosis and chemoresistance/radioresistance in FIGO Ib1-IIa squamous cell cervical cancer. *Cell Tissue Res,* 352**,** 361-9.

CARTEI, G., CARTEI, F., INTERLANDI, G., MENEGHINI, G., JOP, A., ZINGONE, G., TABARO, G. & MAZZOLENI, F. 2000. Oral 5-fluorouracil in squamous cell carcinoma of the skin in the aged. *Am J Clin Oncol,* 23**,** 181-4.

CHANG, C. J., CHAO, C. H., XIA, W., YANG, J. Y., XIONG, Y., LI, C. W., YU, W. H., REHMAN, S. K., HSU, J. L., LEE, H. H., LIU, M., CHEN, C. T., YU, D. & HUNG, M. C. 2011. p53 regulates epithelial-mesenchymal transition and stem cell properties through modulating miRNAs. *Nat Cell Biol,* 13**,** 317-23.

CHANG, K. W., LIU, C. J., CHU, T. H., CHENG, H. W., HUNG, P. S., HU, W. Y. & LIN, S. C. 2008. Association between high miR-211 microRNA expression and the poor prognosis of oral carcinoma. *J Dent Res,* 87**,** 1063-8.

CHAO, C. H., CHANG, C. C., WU, M. J., KO, H. W., WANG, D., HUNG, M. C., YANG, J. Y. & CHANG, C. J. 2014. MicroRNA-205 signaling regulates mammary stem cell fate and tumorigenesis. *J Clin Invest,* 124**,** 3093-106.

CHEN, J., XIAO, H., HUANG, Z., HU, Z., QI, T., ZHANG, B., TAO, X. & LIU, S. H. 2014a. MicroRNA124 regulate cell growth of prostate cancer cells by targeting iASPP. *Int J Clin Exp Pathol,* 7**,** 2283-90.

CHEN, J., XIE, F., ZHANG, L. & JIANG, W. G. 2010. iASPP is over-expressed in human non-small cell lung cancer and regulates the proliferation of lung cancer cells through a p53 associated pathway. *BMC Cancer,* 10**,** 694.

CHEN, Y., YAN, W., HE, S., CHEN, J., CHEN, D., ZHANG, Z., LIU, Z., DING, X. & WANG, A. 2014b. In vitro effect of iASPP on cell growth of oral tongue squamous cell carcinoma. *Chin J Cancer Res,* 26**,** 382-90.

CHIKH, A., MATIN, R. N., SENATORE, V., HUFBAUER, M., LAVERY, D., RAIMONDI, C., OSTANO, P., MELLO-GRAND, M., GHIMENTI, C., BAHTA, A., KHALAF, S., AKGUL, B., BRAUN, K. M., CHIORINO, G., PHILPOTT, M. P., HARWOOD, C. A. & BERGAMASCHI, D. 2011. iASPP/p63 autoregulatory feedback loop is required for the homeostasis of stratified epithelia. *EMBO J,* 30**,** 4261-73.

CHIPUK, J. E., MAURER, U., GREEN, D. R. & SCHULER, M. 2003. Pharmacologic activation of p53 elicits Bax-dependent apoptosis in the absence of transcription. *Cancer Cell,* 4**,** 371-81.

CHU, T. H., YANG, C. C., LIU, C. J., LUI, M. T., LIN, S. C. & CHANG, K. W. 2013. miR-211 promotes the progression of head and neck carcinomas by targeting TGFbetaRII. *Cancer Lett,* 337**,** 115-24.

CLAERHOUT, S., VERSCHOOTEN, L., VAN KELST, S., DE VOS, R., PROBY, C., AGOSTINIS, P. & GARMYN, M. 2010. Concomitant inhibition of AKT and autophagy is required for efficient cisplatin-induced apoptosis of metastatic skin carcinoma. *Int J Cancer,* 127**,** 2790-803.

CLAUSEN, O. P., AASS, H. C., BEIGI, M., PURDIE, K. J., PROBY, C. M., BROWN, V. L., MATTINGSDAL, M., MICCI, F., KOLVRAA, S., BOLUND, L. & DEANGELIS, P. M. 2006. Are keratoacanthomas variants of squamous cell carcinomas? A comparison of chromosomal aberrations by comparative genomic hybridization. *J Invest Dermatol,* 126**,** 2308-15.

CRANMER, L. D., ENGELHARDT, C. & MORGAN, S. S. 2010. Treatment of unresectable and metastatic cutaneous squamous cell carcinoma. *Oncologist,* 15**,** 1320-8.

CRISCIONE, V. D., WEINSTOCK, M. A., NAYLOR, M. F., LUQUE, C., EIDE, M. J., BINGHAM, S. F. & DEPARTMENT OF VETERAN AFFAIRS TOPICAL TRETINOIN CHEMOPREVENTION TRIAL, G. 2009. Actinic keratoses: Natural history and risk of malignant transformation in the Veterans Affairs Topical Tretinoin Chemoprevention Trial. *Cancer,* 115**,** 2523-30.

CROOK, T., NICHOLLS, J. M., BROOKS, L., O'NIONS, J. & ALLDAY, M. J. 2000. High level expression of deltaN-p63: a mechanism for the inactivation of p53 in undifferentiated nasopharyngeal carcinoma (NPC)? *Oncogene,* 19**,** 3439-44.

DAVIS-DUSENBERY, B. N. & HATA, A. 2010. Mechanisms of control of microRNA biogenesis. *J Biochem,* 148**,** 381-92.

DE COLA, A., VOLPE, S., BUDANI, M. C., FERRACIN, M., LATTANZIO, R., TURDO, A., D'AGOSTINO, D., CAPONE, E., STASSI, G., TODARO, M., DI ILIO, C., SALA, G., PIANTELLI, M., NEGRINI, M., VERONESE, A. & DE LAURENZI, V. 2015. miR-205-5p-mediated downregulation of ErbB/HER receptors in breast cancer stem cells results in targeted therapy resistance. *Cell Death Dis,* 6**,** e1823.

DELIC, S., LOTTMANN, N., STELZL, A., LIESENBERG, F., WOLTER, M., GOTZE, S., ZAPATKA, M., SHIIO, Y., SABEL, M. C., FELSBERG, J., REIFENBERGER, G. & RIEMENSCHNEIDER, M. J. 2014. MiR-328 promotes glioma cell invasion via SFRP1-dependent Wnt-signaling activation. *Neuro Oncol,* 16**,** 179-90.

DEYOUNG, M. P. & ELLISEN, L. W. 2007. p63 and p73 in human cancer: defining the network. *Oncogene,* 26**,** 5169-5183.

DEYOUNG, M. P., JOHANNESSEN, C. M., LEONG, C. O., FAQUIN, W., ROCCO, J. W. & ELLISEN, L. W. 2006. Tumor-specific p73 up-regulation mediates p63 dependence in squamous cell carcinoma. *Cancer Res,* 66**,** 9362-9368.

DI COMO, C. J., URIST, M. J., BABAYAN, I., DROBNJAK, M., HEDVAT, C. V., TERUYA-FELDSTEIN, J., POHAR, K., HOOS, A. & CORDON-CARDO, C. 2002. p63 expression profiles in human normal and tumor tissues. *Clin Cancer Res,* 8**,** 494-501.

DICKSON, M. A., HAHN, W. C., INO, Y., RONFARD, V., WU, J. Y., WEINBERG, R. A., LOUIS, D. N., LI, F. P. & RHEINWALD, J. G. 2000. Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. *Mol Cell Biol,* 20**,** 1436-1447.

DIEPGEN, T. L. & MAHLER, V. 2002. The epidemiology of skin cancer. *Br J Dermatol,* 146 Suppl 61**,** 1-6.

DONG, P., IHIRA, K., HAMADA, J., WATARI, H., YAMADA, T., HOSAKA, M., HANLEY, S. J., KUDO, M. & SAKURAGI, N. 2015. Reactivating p53 functions by suppressing its novel inhibitor iASPP: a potential therapeutic opportunity in p53 wild-type tumors. *Oncotarget,* 6**,** 19968-75.

DOTTO, J. E. & GLUSAC, E. J. 2006. p63 is a useful marker for cutaneous spindle cell squamous cell carcinoma. *J Cutan Pathol,* 33**,** 413-7.

DZIUNYCZ, P., IOTZOVA-WEISS, G., ELORANTA, J. J., LAUCHLI, S., HAFNER, J., FRENCH, L. E. & HOFBAUER, G. F. 2010. Squamous cell carcinoma of the skin shows a distinct microRNA profile modulated by UV radiation. *J Invest Dermatol,* 130**,** 2686-9.

DZIUNYCZ, P. J., LEFORT, K., WU, X., FREIBERGER, S. N., NEU, J., DJERBI, N., IOTZOWA-WEISS, G., FRENCH, L. E., DOTTO, G. P. & HOFBAUER, G. F. 2014. The Oncogene ATF3 Is Potentiated by Cyclosporine A and Ultraviolet Light A. *J Invest Dermatol*.

EEDY, D. J. 2000. Non-melanoma skin cancer and the 'new National Health Service': implications for U.K. dermatology? *Br J Dermatol,* 142**,** 397-9.

ESPANEL, X. & SUDOL, M. 2001. Yes-associated protein and p53-binding protein-2 interact through their WW and SH3 domains. *J Biol Chem,* 276**,** 14514-14523.

FASSINA, A., CAPPELLESSO, R., GUZZARDO, V., DALLA VIA, L., PICCOLO, S., VENTURA, L. & FASSAN, M. 2012. Epithelial-mesenchymal transition in malignant mesothelioma. *Mod Pathol,* 25**,** 86-99.

FILIPOWICZ, W., BHATTACHARYYA, S. N. & SONENBERG, N. 2008. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet,* 9**,** 102-14.

FIRE, A., XU, S., MONTGOMERY, M. K., KOSTAS, S. A., DRIVER, S. E. & MELLO, C. C. 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature,* 391**,** 806-11.

FLORES, E. R., SENGUPTA, S., MILLER, J. B., NEWMAN, J. J., BRONSON, R., CROWLEY, D., YANG, A., MCKEON, F. & JACKS, T. 2005. Tumor predisposition in mice mutant for

p63 and p73: evidence for broader tumor suppressor functions for the p53 family. *Cancer Cell,* 7**,** 363-73.

FORBES, S. A., TANG, G., BINDAL, N., BAMFORD, S., DAWSON, E., COLE, C., KOK, C. Y., JIA, M., EWING, R., MENZIES, A., TEAGUE, J. W., STRATTON, M. R. & FUTREAL, P. A. 2010. COSMIC (the Catalogue of Somatic Mutations in Cancer): a resource to investigate acquired mutations in human cancer. *Nucleic Acids Res,* 38**,** D652-7.

FRANKEN, N. A., RODERMOND, H. M., STAP, J., HAVEMAN, J. & VAN BREE, C. 2006. Clonogenic assay of cells in vitro. *Nat Protoc,* 1**,** 2315-9.

FUCHS, E. 1990. Epidermal differentiation: the bare essentials. *J Cell Biol,* 111**,** 2807-14.

FUCHS, E. 2007. Scratching the surface of skin development. *Nature,* 445**,** 834-42.

FUCHS, E. & RAGHAVAN, S. 2002. Getting under the skin of epidermal morphogenesis. *Nat Rev Genet,* 3**,** 199-209.

FUSENIG, N. E., BREITKREUTZ, D., DZARLIEVA, R. T., BOUKAMP, P., BOHNERT, A. & TILGEN, W. 1983. Growth and differentiation characteristics of transformed keratinocytes from mouse and human skin in vitro and in vivo. *J Invest Dermatol,* 81**,** 168s-75s.

GAIDDON, C., LOKSHIN, M., AHN, J., ZHANG, T. & PRIVES, C. 2001. A subset of tumor-derived mutant forms of p53 down-regulate p63 and p73 through a direct interaction with the p53 core domain. *Mol Cell Biol,* 21**,** 1874-87.

GASTALDI, C., BERTERO, T., XU, N., BOURGET-PONZIO, I., LEBRIGAND, K., FOURRE, S., POPA, A., CARDOT-LECCIA, N., MENEGUZZI, G., SONKOLY, E., PIVARCSI, A., MARI, B., BARBRY, P., PONZIO, G. & REZZONICO, R. 2014. miR-193b/365a cluster controls progression of epidermal squamous cell carcinoma. *Carcinogenesis*.

GHIONI, P., BOLOGNESE, F., DUIJF, P. H., VAN BOKHOVEN, H., MANTOVANI, R. & GUERRINI, L. 2002. Complex transcriptional effects of p63 isoforms: identification of novel activation and repression domains. *Mol Cell Biol,* 22**,** 8659-68.

GILLESPIE, J., SKEELES, L. E., ALLAIN, D. C., KENT, M. N., PETERS, S. B., NAGARAJAN, P., YU, L., TEKNOS, T. N., OLENCKI, T. & TOLAND, A. E. 2015. MicroRNA expression profiling in metastatic cutaneous squamous cell carcinoma. *J Eur Acad Dermatol Venereol*.

GILLISON, M. L., CHATURVEDI, A. K. & LOWY, D. R. 2008. HPV prophylactic vaccines and the potential prevention of noncervical cancers in both men and women. *Cancer,* 113**,** 3036-46.

GIOVANNETTI, E., VAN DER VELDE, A., FUNEL, N., VASILE, E., PERRONE, V., LEON, L. G., DE LIO, N., AVAN, A., CAPONI, S., POLLINA, L. E., GALLA, V., SUDO, H., FALCONE, A., CAMPANI, D., BOGGI, U. & PETERS, G. J. 2012. High-throughput microRNA (miRNAs) arrays unravel the prognostic role of MiR-211 in pancreatic cancer. *PLoS One,* 7**,** e49145.

GONFLONI, S., DI TELLA, L., CALDAROLA, S., CANNATA, S. M., KLINGER, F. G., DI BARTOLOMEO, C., MATTEI, M., CANDI, E., DE FELICI, M., MELINO, G. & CESARENI, G. 2009. Inhibition of the c-Abl-TAp63 pathway protects mouse oocytes from chemotherapy-induced death. *Nat Med,* 15**,** 1179-85.

GORINA, S. & PAVLETICH, N. P. 1996. Structure of the p53 tumor suppressor bound to the ankyrin and SH3 domains of 53BP2. *Science,* 274**,** 1001-5.

GREEN, D. R. & KROEMER, G. 2009. Cytoplasmic functions of the tumour suppressor p53. *Nature,* 458**,** 1127-30.

GREGORY, P. A., BERT, A. G., PATERSON, E. L., BARRY, S. C., TSYKIN, A., FARSHID, G., VADAS, M. A., KHEW-GOODALL, Y. & GOODALL, G. J. 2008. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol,* 10**,** 593-601.

GUO, X. C., KEYES, W. M., PAPAZOGLU, C., ZUBER, J., LI, W. Z., LOWE, S. W., VOGEL, H. & MILLS, A. A. 2009. TAp63 induces senescence and suppresses tumorigenesis in vivo. *Nature Cell Biology,* 11**,** 1451-U150.

GUTHRIE, T. H., JR., PORUBSKY, E. S., LUXENBERG, M. N., SHAH, K. J., WURTZ, K. L. & WATSON, P. R. 1990. Cisplatin-based chemotherapy in advanced basal and squamous cell carcinomas of the skin: results in 28 patients including 13 patients receiving multimodality therapy. *J Clin Oncol,* 8**,** 342-6.

GUY, G. P., JR., MACHLIN, S. R., EKWUEME, D. U. & YABROFF, K. R. 2015. Prevalence and costs of skin cancer treatment in the U.S., 2002-2006 and 2007-2011. *Am J Prev Med,* 48**,** 183-7.

GUZMAN, N., AGARWAL, K., ASTHAGIRI, D., YU, L., SAJI, M., RINGEL, M. D. & PAULAITIS, M. E. 2015. Breast Cancer-Specific miR Signature Unique to Extracellular Vesicles Includes "microRNA-like" tRNA Fragments. *Mol Cancer Res,* 13**,** 891-901.

HALL, J. M., SAENGER, J. S. & FADARE, O. 2008. Diagnostic utility of p63 and CD10 in distinguishing cutaneous spindle cell/sarcomatoid squamous cell carcinomas and atypical fibroxanthomas. *Int J Clin Exp Pathol,* 1**,** 524-30.

HARA, E. S., ONO, M., EGUCHI, T., KUBOTA, S., PHAM, H. T., SONOYAMA, W., TAJIMA, S., TAKIGAWA, M., CALDERWOOD, S. K. & KUBOKI, T. 2013. miRNA-720 controls stem cell phenotype, proliferation and differentiation of human dental pulp cells. *PLoS One,* 8**,** e83545.

HARWOOD, C. A., MESHER, D., MCGREGOR, J. M., MITCHELL, L., LEEDHAM-GREEN, M., RAFTERY, M., CERIO, R., LEIGH, I. M., SASIENI, P. & PROBY, C. M. 2013. A surveillance model for skin cancer in organ transplant recipients: a 22-year prospective study in an ethnically diverse population. *Am J Transplant,* 13**,** 119-29.

HARWOOD, C. A. & PROBY, C. M. 2002. Human papillomaviruses and non-melanoma skin cancer. *Curr Opin Infect Dis,* 15**,** 101-14.

HARWOOD, C. A., PROBY, C. M., MCGREGOR, J. M., SHEAFF, M. T., LEIGH, I. M. & CERIO, R. 2006. Clinicopathologic features of skin cancer in organ transplant recipients: a retrospective case-control series. *J Am Acad Dermatol,* 54**,** 290-300.

HAUPT, Y., ROWAN, S., SHAULIAN, E., VOUSDEN, K. H. & OREN, M. 1995. Induction of apoptosis in HeLa cells by trans-activation-deficient p53. *Genes Dev,* 9**,** 2170-83.

HAUSSECKER, D., HUANG, Y., LAU, A., PARAMESWARAN, P., FIRE, A. Z. & KAY, M. A. 2010. Human tRNA-derived small RNAs in the global regulation of RNA silencing. *RNA,* 16**,** 673-95.

HAYFLICK, L. 1965. The Limited in Vitro Lifetime of Human Diploid Cell Strains. *Exp Cell Res,* 37**,** 614-36.

HE, J., YANG, Q. & CHANG, L. J. 2005. Dynamic DNA methylation and histone modifications contribute to lentiviral transgene silencing in murine embryonic carcinoma cells. *J Virol,* 79**,** 13497-508.

HEALY, E. 2004. Melanocortin 1 receptor variants, pigmentation, and skin cancer susceptibility. *Photodermatol Photoimmunol Photomed,* 20**,** 283-8.

HERRON, B. J., RAO, C., LIU, S., LAPRADE, L., RICHARDSON, J. A., OLIVIERI, E., SEMSARIAN, C., MILLAR, S. E., STUBBS, L. & BEIER, D. R. 2005. A mutation in NFkB interacting protein 1 results in cardiomyopathy and abnormal skin development in wa3 mice. *Hum Mol Genet,* 14**,** 667-77.

HESELMEYER, K., SCHROCK, E., DU MANOIR, S., BLEGEN, H., SHAH, K., STEINBECK, R., AUER, G. & RIED, T. 1996. Gain of chromosome 3q defines the transition from severe dysplasia to invasive carcinoma of the uterine cervix. *Proc Natl Acad Sci U S A,* 93**,** 479-84.

HIBI, K., TRINK, B., PATTURAJAN, M., WESTRA, W. H., CABALLERO, O. L., HILL, D. E., RATOVITSKI, E. A., JEN, J. & SIDRANSKY, D. 2000. AIS is an oncogene amplified in squamous cell carcinoma. *Proc Natl Acad Sci U S A,* 97**,** 5462-7.

HUANG, Y. P., SEN, T., NAGPAL, J., UPADHYAY, S., TRINK, B., RATOVITSKI, E. & SIDRANSKY, D. 2008. ATM kinase is a master switch for the Delta Np63 alpha

phosphorylation/degradation in human head and neck squamous cell carcinoma cells upon DNA damage. *Cell Cycle,* 7**,** 2846-2855.

HUIBREGTSE, J. M., SCHEFFNER, M. & HOWLEY, P. M. 1991. A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. *EMBO J,* 10**,** 4129-35.

ISSAEVA, N., BOZKO, P., ENGE, M., PROTOPOPOVA, M., VERHOEF, L. G., MASUCCI, M., PRAMANIK, A. & SELIVANOVA, G. 2004. Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. *Nat Med,* 10**,** 1321-8.

IWABUCHI, K., BARTEL, P. L., LI, B., MARRACCINO, R. & FIELDS, S. 1994. Two cellular proteins that bind to wild-type but not mutant p53. *Proc Natl Acad Sci U S A,* 91**,** 6098-102.

JENEI, V., NYSTROM, M. L. & THOMAS, G. J. 2011. Measuring invasion in an organotypic model. *Methods Mol Biol,* 769**,** 223-32.

JIANG, L., SIU, M. K., WONG, O. G., TAM, K. F., LU, X., LAM, E. W., NGAN, H. Y., LE, X. F., WONG, E. S., MONTEIRO, L. J., CHAN, H. Y. & CHEUNG, A. N. 2011. iASPP and chemoresistance in ovarian cancers: effects on paclitaxel-mediated mitotic catastrophe. *Clin Cancer Res,* 17**,** 6924-33.

JIN, Y., CHEN, Z., LIU, X. & ZHOU, X. 2013. Evaluating the microRNA targeting sites by luciferase reporter gene assay. *Methods Mol Biol,* 936**,** 117-27.

KABEYA, Y., MIZUSHIMA, N., UENO, T., YAMAMOTO, A., KIRISAKO, T., NODA, T., KOMINAMI, E., OHSUMI, Y. & YOSHIMORI, T. 2000. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J,* 19**,** 5720-8.

KALININ, A. E., KAJAVA, A. V. & STEINERT, P. M. 2002. Epithelial barrier function: assembly and structural features of the cornified cell envelope. *Bioessays,* 24**,** 789-800.

KALLURI, R. & WEINBERG, R. A. 2009. The basics of epithelial-mesenchymal transition. *J Clin Invest,* 119**,** 1420-8.

KARGI, A., GUREL, D. & TUNA, B. 2007. The diagnostic value of TTF-1, CK 5/6, and p63 immunostaining in classification of lung carcinomas. *Appl Immunohistochem Mol Morphol,* 15**,** 415-20.

KEYES, W. M., PECORARO, M., ARANDA, V., VERNERSSON-LINDAHL, E., LI, W., VOGEL, H., GUO, X., GARCIA, E. L., MICHURINA, T. V., ENIKOLOPOV, G., MUTHUSWAMY, S. K. & MILLS, A. A. 2011. DeltaNp63alpha is an oncogene that targets chromatin remodeler Lsh to drive skin stem cell proliferation and tumorigenesis. *Cell Stem Cell,* 8**,** 164-76.

KEYES, W. M., WU, Y., VOGEL, H., GUO, X., LOWE, S. W. & MILLS, A. A. 2005. p63 deficiency activates a program of cellular senescence and leads to accelerated aging. *Genes Dev,* 19**,** 1986-99.

KHANSUR, T. & KENNEDY, A. 1991. Cisplatin and 5-fluorouracil for advanced locoregional and metastatic squamous cell carcinoma of the skin. *Cancer,* 67**,** 2030-2.

KHAYYATA, S., YUN, S., PASHA, T., JIAN, B., MCGRATH, C., YU, G., GUPTA, P. & BALOCH, Z. 2009. Value of p63 and CK5/6 in distinguishing squamous cell carcinoma from adenocarcinoma in lung fine-needle aspiration specimens. *Diagn Cytopathol,* 37**,** 178-83.

KIM, B. K., KIM, I. & YOON, S. K. 2015a. Identification of miR-199a-5p target genes in the skin keratinocyte and their expression in cutaneous squamous cell carcinoma. *J Dermatol Sci,* 79**,** 137-47.

KIM, J. W., ROH, J. L., PARK, Y., CHO, K. J., CHOI, S. H., NAM, S. Y. & KIM, S. Y. 2015b. Cytoplasmic iASPP expression as a novel prognostic indicator in oral cavity squamous cell carcinoma. *Ann Surg Oncol,* 22**,** 662-9.

KIM, K., MADAK-ERDOGAN, Z., VENTRELLA, R. & KATZENELLENBOGEN, B. S. 2013. A MicroRNA196a2* and Tp63 circuit regulated by estrogen receptor-alpha and ERK2 that controls breast cancer proliferation and invasiveness properties. *Horm Cancer,* 4**,** 78-91.

KOLEV, V., MANDINOVA, A., GUINEA-VINIEGRA, J., HU, B., LEFORT, K., LAMBERTINI, C., NEEL, V., DUMMER, R., WAGNER, E. F. & DOTTO, G. P. 2008. EGFR signalling as a negative regulator of Notch1 gene transcription and function in proliferating keratinocytes and cancer. *Nat Cell Biol,* 10**,** 902-11.

KOMATSU, S., TAKENOBU, H., OZAKI, T., ANDO, K., KOIDA, N., SUENAGA, Y., ICHIKAWA, T., HISHIKI, T., CHIBA, T., IWAMA, A., YOSHIDA, H., OHNUMA, N., NAKAGAWARA, A. & KAMIJO, T. 2009. Plk1 regulates liver tumor cell death by phosphorylation of TAp63. *Oncogene,* 28**,** 3631-41.

KOSTER, M. I., DAI, D., MARINARI, B., SANO, Y., COSTANZO, A., KARIN, M. & ROOP, D. R. 2007. p63 induces key target genes required for epidermal morphogenesis. *Proc Natl Acad Sci U S A,* 104**,** 3255-60.

KOSTER, M. I., DAI, D. & ROOP, D. R. 2007. Conflicting roles for p63 in skin development and carcinogenesis. *Cell Cycle,* 6**,** 269-73.

KOSTER, M. I. & ROOP, D. R. 2004. Transgenic mouse models provide new insights into the role of p63 in epidermal development. *Cell Cycle,* 3**,** 411-3.

KRAMER, D., SCHON, M., BAYERLOVA, M., BLECKMANN, A., SCHON, M. P., ZORNIG, M. & DOBBELSTEIN, M. 2015. A pro-apoptotic function of iASPP by stabilizing p300 and CBP through inhibition of BRMS1 E3 ubiquitin ligase activity. *Cell Death Dis,* 6**,** e1634.

LAMOUILLE, S., XU, J. & DERYNCK, R. 2014. Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol,* 15**,** 178-96.

LANZA, M., MARINARI, B., PAPOUTSAKI, M., GIUSTIZIERI, M. L., D'ALESSANDRA, Y., CHIMENTI, S., GUERRINI, L. & COSTANZO, A. 2006. Cross-talks in the p53 family: deltaNp63 is an anti-apoptotic target for deltaNp73alpha and p53 gain-of-function mutants. *Cell Cycle,* 5**,** 1996-2004.

LASKA, M. J., STRANDBYGARD, D., KJELDGAARD, A., MAINS, M., CORYDON, T. J., MEMON, A. A., SORENSEN, B. S., VOGEL, U., JENSEN, U. B. & NEXO, B. A. 2007. Expression of the RAI gene is conducive to apoptosis: studies of induction and interference. *Exp Cell Res,* 313**,** 2611-21.

LAURIKKALA, J., MIKKOLA, M. L., JAMES, M., TUMMERS, M., MILLS, A. A. & THESLEFF, I. 2006. p63 regulates multiple signalling pathways required for ectodermal organogenesis and differentiation. *Development,* 133**,** 1553-63.

LEE, H., JUN, S. Y., LEE, Y. S., LEE, H. J., LEE, W. S. & PARK, C. S. 2014a. Expression of miRNAs and ZEB1 and ZEB2 correlates with histopathological grade in papillary urothelial tumors of the urinary bladder. *Virchows Arch,* 464**,** 213-20.

LEE, J. Y., PARK, M. K., PARK, J. H., LEE, H. J., SHIN, D. H., KANG, Y., LEE, C. H. & KONG, G. 2014b. Loss of the polycomb protein Mel-18 enhances the epithelial-mesenchymal transition by ZEB1 and ZEB2 expression through the downregulation of miR-205 in breast cancer. *Oncogene,* 33**,** 1325-35.

LEIDINGER, P., KELLER, A., BORRIES, A., REICHRATH, J., RASS, K., JAGER, S. U., LENHOF, H. P. & MEESE, E. 2010. High-throughput miRNA profiling of human melanoma blood samples. *BMC Cancer,* 10**,** 262.

LENA, A. M., SHALOM-FEUERSTEIN, R., RIVETTI DI VAL CERVO, P., ABERDAM, D., KNIGHT, R. A., MELINO, G. & CANDI, E. 2008. miR-203 represses 'stemness' by repressing DeltaNp63. *Cell Death Differ,* 15**,** 1187-95.

LEVY, C., KHALED, M., ILIOPOULOS, D., JANAS, M. M., SCHUBERT, S., PINNER, S., CHEN, P. H., LI, S., FLETCHER, A. L., YOKOYAMA, S., SCOTT, K. L., GARRAWAY, L. A., SONG, J. S., GRANTER, S. R., TURLEY, S. J., FISHER, D. E. & NOVINA, C. D. 2010. Intronic miR-211 assumes the tumor suppressive function of its host gene in melanoma. *Mol Cell,* 40**,** 841-9.

LEWIS, C. M., GLISSON, B. S., FENG, L., WAN, F., TANG, X., WISTUBA, II, EL-NAGGAR, A. K., ROSENTHAL, D. I., CHAMBERS, M. S., LUSTIG, R. A. & WEBER, R. S. 2012. A phase II

study of gefitinib for aggressive cutaneous squamous cell carcinoma of the head and neck. *Clin Cancer Res,* 18**,** 1435-46.

LEWIS, J. S., RITTER, J. H. & EL-MOFTY, S. 2005. Alternative epithelial markers in sarcomatoid carcinomas of the head and neck, lung, and bladder-p63, MOC-31, and TTF-1. *Mod Pathol,* 18**,** 1471-81.

LEWIS, M. P., LYGOE, K. A., NYSTROM, M. L., ANDERSON, W. P., SPEIGHT, P. M., MARSHALL, J. F. & THOMAS, G. J. 2004. Tumour-derived TGF-beta1 modulates myofibroblast differentiation and promotes HGF/SF-dependent invasion of squamous carcinoma cells. *Br J Cancer,* 90**,** 822-32.

LI, G., WANG, R., GAO, J., DENG, K., WEI, J. & WEI, Y. 2011. RNA interference-mediated silencing of iASPP induces cell proliferation inhibition and G0/G1 cell cycle arrest in U251 human glioblastoma cells. *Mol Cell Biochem,* 350**,** 193-200.

LI, J. R., WANG, J. Q., GONG, Q., FANG, R. H. & GUO, Y. L. 2015. MicroRNA-328 inhibits proliferation of human melanoma cells by targeting TGFbeta2. *Asian Pac J Cancer Prev,* 16**,** 1575-9.

LI, Y. & PRIVES, C. 2007. Are interactions with p63 and p73 involved in mutant p53 gain of oncogenic function? *Oncogene,* 26**,** 2220-5.

LI, Y., ZHOU, Z., ALIMANDI, M. & CHEN, C. 2009. WW domain containing E3 ubiquitin protein ligase 1 targets the full-length ErbB4 for ubiquitin-mediated degradation in breast cancer. *Oncogene,* 28**,** 2948-58.

LIEFER, K. M., KOSTER, M. I., WANG, X. J., YANG, A., MCKEON, F. & ROOP, D. R. 2000. Down-regulation of p63 is required for epidermal UV-B-induced apoptosis. *Cancer Res,* 60**,** 4016-20.

LIN, B. L., XIE, D. Y., XIE, S. B., XIE, J. Q., ZHANG, X. H., ZHANG, Y. F. & GAO, Z. L. 2011. Down-regulation of iASPP in human hepatocellular carcinoma cells inhibits cell proliferation and tumor growth. *Neoplasma,* 58**,** 205-10.

LIN, C., LI, X., ZHANG, Y., GUO, Y., ZHOU, J., GAO, K., DAI, J., HU, G., LV, L., DU, J. & ZHANG, Y. 2015. The microRNA feedback regulation of p63 in cancer progression. *Oncotarget,* 6**,** 8434-53.

LIOTTA, L. A., TRYGGVASON, K., GARBISA, S., HART, I., FOLTZ, C. M. & SHAFIE, S. 1980. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature,* 284**,** 67-8.

LIU, H., WANG, M., DIAO, S., RAO, Q., ZHANG, X., XING, H. & WANG, J. 2009. siRNA-mediated down-regulation of iASPP promotes apoptosis induced by etoposide and daunorubicin in leukemia cells expressing wild-type p53. *Leuk Res,* 33**,** 1243-8.

LIU, K., ZHAO, H., YAO, H., LEI, S., LEI, Z., LI, T. & QI, H. 2013a. MicroRNA-124 regulates the proliferation of colorectal cancer cells by targeting iASPP. *Biomed Res Int,* 2013**,** 867537.

LIU, T., LI, L., YANG, W., JIA, H., XU, M., BI, J., LI, Z., LIU, X., LI, Z., JING, H. & KONG, C. 2011. iASPP is important for bladder cancer cell proliferation. *Oncol Res,* 19**,** 125-30.

LIU, W. K., JIANG, X. Y., REN, J. K. & ZHANG, Z. X. 2010. Expression pattern of the ASPP family members in endometrial endometrioid adenocarcinoma. *Onkologie,* 33**,** 500-3.

LIU, X., LI, F., ZHAO, S., LUO, Y., KANG, J., ZHAO, H., YAN, F., LI, S. & JI, X. 2013b. MicroRNA-124-mediated regulation of inhibitory member of apoptosis-stimulating protein of p53 family in experimental stroke. *Stroke,* 44**,** 1973-80.

LIU, Z., ZHANG, X., HUANG, D., LIU, Y., ZHANG, X., LIU, L., LI, G., DAI, Y., TAN, H., XIAO, J. & TIAN, Y. 2012. Elevated expression of iASPP in head and neck squamous cell carcinoma and its clinical significance. *Med Oncol,* 29**,** 3381-8.

LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods,* 25**,** 402-8.

LU, B., GUO, H., ZHAO, J., WANG, C., WU, G., PANG, M., TONG, X., BU, F., LIANG, A., HOU, S., FAN, X., DAI, J., WANG, H. & GUO, Y. 2010. Increased expression of iASPP, regulated by hepatitis B virus X protein-mediated NF-kappaB activation, in hepatocellular carcinoma. *Gastroenterology,* 139**,** 2183-2194 e5.

LU, M., BREYSSENS, H., SALTER, V., ZHONG, S., HU, Y., BAER, C., RATNAYAKA, I., SULLIVAN, A., BROWN, N. R., ENDICOTT, J., KNAPP, S., KESSLER, B. M., MIDDLETON, M. R., SIEBOLD, C., JONES, E. Y., SVIDERSKAYA, E. V., CEBON, J., JOHN, T., CABALLERO, O. L., GODING, C. R. & LU, X. 2013. Restoring p53 function in human melanoma cells by inhibiting MDM2 and cyclin B1/CDK1-phosphorylated nuclear iASPP. *Cancer Cell,* 23**,** 618-33.

LU, M., ZAK, J., CHEN, S., SANCHEZ-PULIDO, L., SEVERSON, D. T., ENDICOTT, J., PONTING, C. P., SCHOFIELD, C. J. & LU, X. 2014. A code for RanGDP binding in ankyrin repeats defines a nuclear import pathway. *Cell,* 157**,** 1130-45.

LUO, W. R., LI, S. Y., CAI, L. M. & YAO, K. T. 2012. High expression of nuclear Snail, but not cytoplasmic staining, predicts poor survival in nasopharyngeal carcinoma. *Ann Surg Oncol,* 19**,** 2971-9.

MACPARTLIN, M., ZENG, S. X. & LU, H. 2008. Phosphorylation and stabilization of TAp63gamma by IkappaB kinase-beta. *J Biol Chem,* 283**,** 15754-61.

MADAN, V., LEAR, J. T. & SZEIMIES, R. M. 2010. Non-melanoma skin cancer. *Lancet,* 375**,** 673-85.

MAFTOUH, M., AVAN, A., FUNEL, N., FRAMPTON, A. E., FIUJI, H., PELLICCIONI, S., CASTELLANO, L., GALLA, V., PETERS, G. J. & GIOVANNETTI, E. 2014. miR-211 modulates gemcitabine activity through downregulation of ribonucleotide reductase and inhibits the invasive behavior of pancreatic cancer cells. *Nucleosides Nucleotides Nucleic Acids,* 33**,** 384-93.

MANCA, S., MAGRELLI, A., CIALFI, S., LEFORT, K., AMBRA, R., ALIMANDI, M., BIOLCATI, G., UCCELLETTI, D., PALLESCHI, C., SCREPANTI, I., CANDI, E., MELINO, G., SALVATORE, M., TARUSCIO, D. & TALORA, C. 2011. Oxidative stress activation of miR-125b is part of the molecular switch for Hailey-Hailey disease manifestation. *Exp Dermatol,* 20**,** 932-7.

MANGIULLI, M., VALLETTI, A., CARATOZZOLO, M. F., TULLO, A., SBISA, E., PESOLE, G. & D'ERCHIA, A. M. 2009. Identification and functional characterization of two new transcriptional variants of the human p63 gene. *Nucleic Acids Res,* 37**,** 6092-104.

MANNI, I., ARTUSO, S., CARECCIA, S., RIZZO, M. G., BASERGA, R., PIAGGIO, G. & SACCHI, A. 2009. The microRNA miR-92 increases proliferation of myeloid cells and by targeting p63 modulates the abundance of its isoforms. *FASEB J,* 23**,** 3957-66.

MARCEL, V., PETIT, I., MURRAY-ZMIJEWSKI, F., GOULLET DE RUGY, T., FERNANDES, K., MEURAY, V., DIOT, A., LANE, D. P., ABERDAM, D. & BOURDON, J. C. 2012. Diverse p63 and p73 isoforms regulate Delta133p53 expression through modulation of the internal Tp53 promoter activity. *Cell Death Differ,* 19**,** 816-26.

MARSHALL, J. 2011. Transwell((R)) invasion assays. *Methods Mol Biol,* 769**,** 97-110.

MASSION, P. P., TAFLAN, P. M., JAMSHEDUR RAHMAN, S. M., YILDIZ, P., SHYR, Y., EDGERTON, M. E., WESTFALL, M. D., ROBERTS, J. R., PIETENPOL, J. A., CARBONE, D. P. & GONZALEZ, A. L. 2003. Significance of p63 amplification and overexpression in lung cancer development and prognosis. *Cancer Res,* 63**,** 7113-21.

MATIN, R. N., CHIKH, A., CHONG, S. L., MESHER, D., GRAF, M., SANZA, P., SENATORE, V., SCATOLINI, M., MORETTI, F., LEIGH, I. M., PROBY, C. M., COSTANZO, A., CHIORINO, G., CERIO, R., HARWOOD, C. A. & BERGAMASCHI, D. 2013. p63 is an alternative p53 repressor in melanoma that confers chemoresistance and a poor prognosis. *J Exp Med,* 210**,** 581-603.

MATTICK, J. S. & MAKUNIN, I. V. 2005. Small regulatory RNAs in mammals. *Hum Mol Genet,* 14 Spec No 1**,** R121-32.

MAUBEC, E., DUVILLARD, P., VELASCO, V., CRICKX, B. & AVRIL, M. F. 2005. Immunohistochemical analysis of EGFR and HER-2 in patients with metastatic squamous cell carcinoma of the skin. *Anticancer Res,* 25**,** 1205-10.

MAUBEC, E., PETROW, P., SCHEER-SENYARICH, I., DUVILLARD, P., LACROIX, L., GELLY, J., CERTAIN, A., DUVAL, X., CRICKX, B., BUFFARD, V., BASSET-SEGUIN, N., SAEZ, P., DUVAL-MODESTE, A. B., ADAMSKI, H., MANSARD, S., GRANGE, F., DOMPMARTIN, A., FAIVRE, S., MENTRE, F. & AVRIL, M. F. 2011. Phase II study of cetuximab as first-line single-drug therapy in patients with unresectable squamous cell carcinoma of the skin. *J Clin Oncol,* 29**,** 3419-26.

MAZAR, J., DEYOUNG, K., KHAITAN, D., MEISTER, E., ALMODOVAR, A., GOYDOS, J., RAY, A. & PERERA, R. J. 2010. The regulation of miRNA-211 expression and its role in melanoma cell invasiveness. *PLoS One,* 5**,** e13779.

MILLS, A. A., ZHENG, B., WANG, X. J., VOGEL, H., ROOP, D. R. & BRADLEY, A. 1999. p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature,* 398**,** 708-13.

MIZUSHIMA, N., YOSHIMORI, T. & OHSUMI, Y. 2011. The role of Atg proteins in autophagosome formation. *Annu Rev Cell Dev Biol,* 27**,** 107-32.

MOORE, C. B., GUTHRIE, E. H., HUANG, M. T. & TAXMAN, D. J. 2010. Short hairpin RNA (shRNA): design, delivery, and assessment of gene knockdown. *Methods Mol Biol,* 629**,** 141-58.

MORRIS, E. V., CERUNDOLO, L., LU, M., VERRILL, C., FRITZSCHE, F., WHITE, M. J., THALMANN, G. N., TEN DONKELAAR, C. S., RATNAYAKA, I., SALTER, V., HAMDY, F. C., LU, X. & BRYANT, R. J. 2014. Nuclear iASPP may facilitate prostate cancer progression. *Cell Death Dis,* 5**,** e1492.

MURRAY-ZMIJEWSKI, F., LANE, D. P. & BOURDON, J. C. 2006. p53/p63/p73 isoforms: an orchestra of isoforms to harmonise cell differentiation and response to stress. *Cell Death Differ,* 13**,** 962-72.

NAIK, E., MICHALAK, E. M., VILLUNGER, A., ADAMS, J. M. & STRASSER, A. 2007. Ultraviolet radiation triggers apoptosis of fibroblasts and skin keratinocytes mainly via the BH3-only protein Noxa. *J Cell Biol,* 176**,** 415-24.

NGUYEN, B. C., LEFORT, K., MANDINOVA, A., ANTONINI, D., DEVGAN, V., DELLA GATTA, G., KOSTER, M. I., ZHANG, Z., WANG, J., DI VIGNANO, A. T., KITAJEWSKI, J., CHIORINO, G., ROOP, D. R., MISSERO, C. & DOTTO, G. P. 2006. Cross-regulation between Notch and p63 in keratinocyte commitment to differentiation. *Genes & Development,* 20**,** 1028-1042.

NIU, K., SHEN, W., ZHANG, Y., ZHAO, Y. & LU, Y. 2015. MiR-205 promotes motility of ovarian cancer cells via targeting ZEB1. *Gene*.

NOTARI, M., HU, Y., KOCH, S., LU, M., RATNAYAKA, I., ZHONG, S., BAER, C., PAGOTTO, A., GOLDIN, R., SALTER, V., CANDI, E., MELINO, G. & LU, X. 2011. Inhibitor of apoptosis-stimulating protein of p53 (iASPP) prevents senescence and is required for epithelial stratification. *Proc Natl Acad Sci U S A,* 108**,** 16645-50.

NOTARI, M., HU, Y., SUTENDRA, G., DEDEIC, Z., LU, M., DUPAYS, L., YAVARI, A., CARR, C. A., ZHONG, S., OPEL, A., TINKER, A., CLARKE, K., WATKINS, H., FERGUSON, D. J., KELSELL, D. P., DE NORONHA, S., SHEPPARD, M. N., HOLLINSHEAD, M., MOHUN, T. J. & LU, X. 2015. iASPP, a previously unidentified regulator of desmosomes, prevents arrhythmogenic right ventricular cardiomyopathy (ARVC)-induced sudden death. *Proc Natl Acad Sci U S A,* 112**,** E973-81.

NYSTROM, M. L., THOMAS, G. J., STONE, M., MACKENZIE, I. C., HART, I. R. & MARSHALL, J. F. 2005. Development of a quantitative method to analyse tumour cell invasion in organotypic culture. *J Pathol,* 205**,** 468-75.

OCQUE, R., TOCHIGI, N., OHORI, N. P. & DACIC, S. 2011. Usefulness of immunohistochemical and histochemical studies in the classification of lung adenocarcinoma and squamous cell carcinoma in cytologic specimens. *Am J Clin Pathol,* 136**,** 81-7.

O'DONOVAN, P., PERRETT, C. M., ZHANG, X., MONTANER, B., XU, Y. Z., HARWOOD, C. A., MCGREGOR, J. M., WALKER, S. L., HANAOKA, F. & KARRAN, P. 2005. Azathioprine and UVA light generate mutagenic oxidative DNA damage. *Science,* 309**,** 1871-4.

OH, J. E., KIM, R. H., SHIN, K. H., PARK, N. H. & KANG, M. K. 2011. DeltaNp63alpha protein triggers epithelial-mesenchymal transition and confers stem cell properties in normal human keratinocytes. *J Biol Chem,* 286**,** 38757-67.

OLASZ, E. B., SELINE, L. N., SCHOCK, A. M., DUNCAN, N. E., LOPEZ, A., LAZAR, J., FLISTER, M. J., LU, Y., LIU, P., SOKUMBI, O., HARWOOD, C. A., PROBY, C. M., NEUBURG, M. & LAZAROVA, Z. 2015. MicroRNA-135b Regulates Leucine Zipper Tumor Suppressor 1 in Cutaneous Squamous Cell Carcinoma. *PLoS One,* 10**,** e0125412.

PANDOLFI, S., MONTAGNANI, V., LAPUCCI, A. & STECCA, B. 2015. HEDGEHOG/GLI-E2F1 axis modulates iASPP expression and function and regulates melanoma cell growth. *Cell Death Differ*.

PANG, M. S., CHEN, X., LU, B., ZHAO, J., LI, B. H., WEI, Y. Q. & GUO, Y. J. 2010. Lentiviral vector-mediated doxycycline-inducible iASPP gene targeted RNA interference in hepatocellular carcinoma. *Chin J Cancer,* 29**,** 796-801.

PAPAGIANNAKOPOULOS, T., SHAPIRO, A. & KOSIK, K. S. 2008. MicroRNA-21 targets a network of key tumor-suppressive pathways in glioblastoma cells. *Cancer Res,* 68**,** 8164-72.

PARK, B. J., LEE, S. J., KIM, J. I., LEE, S. J., LEE, C. H., CHANG, S. G., PARK, J. H. & CHI, S. G. 2000. Frequent alteration of p63 expression in human primary bladder carcinomas. *Cancer Res,* 60**,** 3370-3374.

PARSA, R., YANG, A., MCKEON, F. & GREEN, H. 1999. Association of p63 with proliferative potential in normal and neoplastic human keratinocytes. *J Invest Dermatol,* 113**,** 1099-105.

PELISSON, I., SOLER, C., CHARDONNET, Y., EUVRARD, S. & SCHMITT, D. 1996. A possible role for human papillomaviruses and c-myc, c-Ha-ras, and p53 gene alterations in malignant cutaneous lesions from renal transplant recipients. *Cancer Detect Prev,* 20**,** 20-30.

PFEIFER, G. P., YOU, Y. H. & BESARATINIA, A. 2005. Mutations induced by ultraviolet light. *Mutat Res,* 571**,** 19-31.

PILLAI, S., BIKLE, D. D., HINCENBERGS, M. & ELIAS, P. M. 1988. Biochemical and morphological characterization of growth and differentiation of normal human neonatal keratinocytes in a serum-free medium. *J Cell Physiol,* 134**,** 229-37.

PINTO, E. M., MUSOLINO, N. R., CESCATO, V. A., SOARES, I. C., WAKAMATSU, A., DE OLIVEIRA, E., SALGADO, L. R. & BRONSTEIN, M. D. 2010. iASPP: a novel protein involved in pituitary tumorigenesis? *Front Horm Res,* 38**,** 70-6.

PONTICELLI, C., CUCCHIARI, D. & BENCINI, P. 2014. Skin cancer in kidney transplant recipients. *J Nephrol,* 27**,** 385-94.

POPP, S., WALTERING, S., HOLTGREVE-GREZ, H., JAUCH, A., PROBY, C., LEIGH, I. M. & BOUKAMP, P. 2000. Genetic characterization of a human skin carcinoma progression model: from primary tumor to metastasis. *J Invest Dermatol,* 115**,** 1095-103.

PRIVES, C. & HALL, P. A. 1999. The p53 pathway. *J Pathol,* 187**,** 112-26.

PROKSCH, E., BRANDNER, J. M. & JENSEN, J. M. 2008. The skin: an indispensable barrier. *Exp Dermatol,* 17**,** 1063-72.

PURDIE, K. J., HARWOOD, C. A., GULATI, A., CHAPLIN, T., LAMBERT, S. R., CERIO, R., KELLY, G. P., CAZIER, J. B., YOUNG, B. D., LEIGH, I. M. & PROBY, C. M. 2009. Single nucleotide polymorphism array analysis defines a specific genetic fingerprint for well-differentiated cutaneous SCCs. *J Invest Dermatol,* 129**,** 1562-8.

PURDIE, K. J., LAMBERT, S. R., TEH, M. T., CHAPLIN, T., MOLLOY, G., RAGHAVAN, M., KELSELL, D. P., LEIGH, I. M., HARWOOD, C. A., PROBY, C. M. & YOUNG, B. D. 2007. Allelic imbalances and microdeletions affecting the PTPRD gene in cutaneous squamous cell carcinomas detected using single nucleotide polymorphism microarray analysis. *Genes Chromosomes Cancer,* 46**,** 661-9.

QIU, S., CAI, Y., GAO, X., GU, S. Z. & LIU, Z. J. 2015. A small peptide derived from p53 linker region can resume the apoptotic activity of p53 by sequestering iASPP with p53. *Cancer Lett,* 356**,** 910-7.

QUINN, A. G., CAMPBELL, C., HEALY, E. & REES, J. L. 1994a. Chromosome 9 allele loss occurs in both basal and squamous cell carcinomas of the skin. *J Invest Dermatol,* 102**,** 300-3.

QUINN, A. G., SIKKINK, S. & REES, J. L. 1994b. Basal cell carcinomas and squamous cell carcinomas of human skin show distinct patterns of chromosome loss. *Cancer Res,* 54**,** 4756-9.

QUINTAVALLE, C., DONNARUMMA, E., IABONI, M., ROSCIGNO, G., GAROFALO, M., ROMANO, G., FIORE, D., DE MARINIS, P., CROCE, C. M. & CONDORELLI, G. 2013. Effect of miR-21 and miR-30b/c on TRAIL-induced apoptosis in glioma cells. *Oncogene,* 32**,** 4001-8.

RATOVITSKI, E. A., PATTURAJAN, M., HIBI, K., TRINK, B., YAMAGUCHI, K. & SIDRANSKY, D. 2001. p53 associates with and targets Delta Np63 into a protein degradation pathway. *Proc Natl Acad Sci U S A,* 98**,** 1817-22.

RATUSHNY, V., GOBER, M. D., HICK, R., RIDKY, T. W. & SEYKORA, J. T. 2012. From keratinocyte to cancer: the pathogenesis and modeling of cutaneous squamous cell carcinoma. *J Clin Invest,* 122**,** 464-72.

REIS-FILHO, J. S., TORIO, B., ALBERGARIA, A. & SCHMITT, F. C. 2002. p63 expression in normal skin and usual cutaneous carcinomas. *J Cutan Pathol,* 29**,** 517-23.

RESTIVO, G., NGUYEN, B. C., DZIUNYCZ, P., RISTORCELLI, E., RYAN, R. J. H., OZUYSAL, O. Y., DI PIAZZA, M., RADTKE, F., DIXON, M. J., HOFBAUER, G. F. L., LEFORT, K. & DOTTO, G. P. 2011. IRF6 is a mediator of Notch pro-differentiation and tumour suppressive function in keratinocytes. *Embo Journal,* 30**,** 4571-4585.

RETTIG, E. M., CHUNG, C. H., BISHOP, J. A., HOWARD, J. D., SHARMA, R., LI, R. J., DOUVILLE, C., KARCHIN, R., IZUMCHENKO, E., SIDRANSKY, D., KOCH, W., CALIFANO, J., AGRAWAL, N. & FAKHRY, C. 2015. Cleaved NOTCH1 Expression Pattern in Head and Neck Squamous Cell Carcinoma Is Associated with NOTCH1 Mutation, HPV Status, and High-Risk Features. *Cancer Prev Res (Phila),* 8**,** 287-95.

REYNOLDS, A., LEAKE, D., BOESE, Q., SCARINGE, S., MARSHALL, W. S. & KHVOROVA, A. 2004. Rational siRNA design for RNA interference. *Nat Biotechnol,* 22**,** 326-30.

RINNE, T., BRUNNER, H. G. & VAN BOKHOVEN, H. 2007. p63-associated disorders. *Cell Cycle,* 6**,** 262-268.

RIVETTI DI VAL CERVO, P., LENA, A. M., NICOLOSO, M., ROSSI, S., MANCINI, M., ZHOU, H., SAINTIGNY, G., DELLAMBRA, E., ODORISIO, T., MAHE, C., CALIN, G. A., CANDI, E. & MELINO, G. 2012. p63-microRNA feedback in keratinocyte senescence. *Proc Natl Acad Sci U S A,* 109**,** 1133-8.

ROBINSON, R. A., LU, X., JONES, E. Y. & SIEBOLD, C. 2008. Biochemical and structural studies of ASPP proteins reveal differential binding to p53, p63, and p73. *Structure,* 16**,** 259-68.

ROCCO, J. W., LEONG, C. O., KUPERWASSER, N., DEYOUNG, M. P. & ELLISEN, L. W. 2006. p63 mediates survival in squamous cell carcinoma by suppression of p73-dependent apoptosis. *Cancer Cell,* 9**,** 45-56.

ROGERS, H. W., WEINSTOCK, M. A., FELDMAN, S. R. & COLDIRON, B. M. 2015. Incidence Estimate of Nonmelanoma Skin Cancer (Keratinocyte Carcinomas) in the US Population, 2012. *JAMA Dermatol,* 151**,** 1081-6.

ROSENBLUTH, J. M., JOHNSON, K., TANG, L., TRIPLETT, T. & PIETENPOL, J. A. 2009. Evaluation of p63 and p73 antibodies for cross-reactivity. *Cell Cycle,* 8**,** 3702-6.

ROSSI, M., AQEILAN, R. I., NEALE, M., CANDI, E., SALOMONI, P., KNIGHT, R. A., CROCE, C. M. & MELINO, G. 2006. The E3 ubiquitin ligase Itch controls the protein stability of p63. *Proc Natl Acad Sci U S A,* 103**,** 12753-8.

SADEK, H., AZLI, N., WENDLING, J. L., CVITKOVIC, E., RAHAL, M., MAMELLE, G., GUILLAUME, J. C., ARMAND, J. P. & AVRIL, M. F. 1990. Treatment of advanced squamous cell carcinoma of the skin with cisplatin, 5-fluorouracil, and bleomycin. *Cancer,* 66**,** 1692-6.

SAEBO, M., SKJELBRED, C. F., NEXO, B. A., WALLIN, H., HANSTEEN, I. L., VOGEL, U. & KURE, E. H. 2006. Increased mRNA expression levels of ERCC1, OGG1 and RAI in colorectal adenomas and carcinomas. *BMC Cancer,* 6**,** 208.

SAKURAI, E., MAESAWA, C., SHIBAZAKI, M., YASUHIRA, S., OIKAWA, H., SATO, M., TSUNODA, K., ISHIKAWA, Y., WATANABE, A., TAKAHASHI, K., AKASAKA, T. & MASUDA, T. 2011. Downregulation of microRNA-211 is involved in expression of preferentially expressed antigen of melanoma in melanoma cells. *Int J Oncol,* 39**,** 665-72.

SAMARASINGHE, V. & MADAN, V. 2012. Nonmelanoma skin cancer. *J Cutan Aesthet Surg,* 5**,** 3-10.

SAMARASINGHE, V., MADAN, V. & LEAR, J. T. 2011. Management of high-risk squamous cell carcinoma of the skin. *Expert Rev Anticancer Ther,* 11**,** 763-9.

SAMUELS-LEV, Y., O'CONNOR, D. J., BERGAMASCHI, D., TRIGIANTE, G., HSIEH, J. K., ZHONG, S., CAMPARGUE, I., NAUMOVSKI, L., CROOK, T. & LU, X. 2001. ASPP proteins specifically stimulate the apoptotic function of p53. *Mol Cell,* 8**,** 781-94.

SANCHEZ-TILLO, E., LAZARO, A., TORRENT, R., CUATRECASAS, M., VAQUERO, E. C., CASTELLS, A., ENGEL, P. & POSTIGO, A. 2010. ZEB1 represses E-cadherin and induces an EMT by recruiting the SWI/SNF chromatin-remodeling protein BRG1. *Oncogene,* 29**,** 3490-500.

SAND, M., SKRYGAN, M., GEORGAS, D., SAND, D., HAHN, S. A., GAMBICHLER, T., ALTMEYER, P. & BECHARA, F. G. 2012. Microarray analysis of microRNA expression in cutaneous squamous cell carcinoma. *J Dermatol Sci,* 68**,** 119-26.

SARIDAKI, Z., LILOGLOU, T., ZAFIROPOULOS, A., KOUMANTAKI, E., ZORAS, O. & SPANDIDOS, D. A. 2003. Mutational analysis of CDKN2A genes in patients with squamous cell carcinoma of the skin. *Br J Dermatol,* 148**,** 638-48.

SBISA, E., MASTROPASQUA, G., LEFKIMMIATIS, K., CARATOZZOLO, M. F., D'ERCHIA, A. M. & TULLO, A. 2006. Connecting p63 to cellular proliferation: the example of the adenosine deaminase target gene. *Cell Cycle,* 5**,** 205-12.

SCHADENDORF, D. & HAUSCHILD, A. 2014. Melanoma in 2013: Melanoma--the run of success continues. *Nat Rev Clin Oncol,* 11**,** 75-6.

SCHEEL, A. H., BEYER, U., AGAMI, R. & DOBBELSTEIN, M. 2009. Immunofluorescence-based screening identifies germ cell associated microRNA 302 as an antagonist to p63 expression. *Cell Cycle,* 8**,** 1426-32.

SCHEFFNER, M., WERNESS, B. A., HUIBREGTSE, J. M., LEVINE, A. J. & HOWLEY, P. M. 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell,* 63**,** 1129-36.

SCHOPMAN, N. C., HEYNEN, S., HAASNOOT, J. & BERKHOUT, B. 2010. A miRNA-tRNA mix-up: tRNA origin of proposed miRNA. *RNA Biol,* 7**,** 573-6.

SENOO, M., TSUCHIYA, I., MATSUMURA, Y., MORI, T., SAITO, Y., KATO, H., OKAMOTO, T. & HABU, S. 2001. Transcriptional dysregulation of the p73L / p63 / p51 / p40 / KET gene in human squamous cell carcinomas: expression of Delta Np73L, a novel dominant-negative isoform, and loss of expression of the potential tumour suppressor p51. *Br J Cancer,* 84**,** 1235-41.

SHARIFI, M., SALEHI, R., GHEISARI, Y. & KAZEMI, M. 2014. Inhibition of microRNA miR-92a induces apoptosis and inhibits cell proliferation in human acute promyelocytic leukemia through modulation of p63 expression. *Mol Biol Rep*.

SIDDIK, Z. H. 2003. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene,* 22**,** 7265-79.

SIMPSON, M. A., COOK, R. W., SOLANKI, P., PATTON, M. A., DENNIS, J. A. & CROSBY, A. H. 2009. A mutation in NFkappaB interacting protein 1 causes cardiomyopathy and woolly haircoat syndrome of Poll Hereford cattle. *Anim Genet,* 40**,** 42-6.

SLEE, E. A., GILLOTIN, S., BERGAMASCHI, D., ROYER, C., LLANOS, S., ALI, S., JIN, B., TRIGIANTE, G. & LU, X. 2004. The N-terminus of a novel isoform of human iASPP is required for its cytoplasmic localization. *Oncogene,* 23**,** 9007-16.

SOMMER, M., POLIAK, N., UPADHYAY, S., RATOVITSKI, E., NELKIN, B. D., DONEHOWER, L. A. & SIDRANSKY, D. 2006. DeltaNp63alpha overexpression induces downregulation of Sirt1 and an accelerated aging phenotype in the mouse. *Cell Cycle,* 5**,** 2005-11.

SONG, H. & YANG, P. C. 2010. Construction of shRNA lentiviral vector. *N Am J Med Sci,* 2**,** 598-601.

SOUTH, A. P., CHO, R. J. & ASTER, J. C. 2012. The double-edged sword of Notch signaling in cancer. *Semin Cell Dev Biol,* 23**,** 458-64.

STECCA, B., MAS, C., CLEMENT, V., ZBINDEN, M., CORREA, R., PIGUET, V., BEERMANN, F. & RUIZ, I. A. A. 2007. Melanomas require HEDGEHOG-GLI signaling regulated by interactions between GLI1 and the RAS-MEK/AKT pathways. *Proc Natl Acad Sci U S A,* 104**,** 5895-900.

STRANSKY, N., EGLOFF, A. M., TWARD, A. D., KOSTIC, A. D., CIBULSKIS, K., SIVACHENKO, A., KRYUKOV, G. V., LAWRENCE, M. S., SOUGNEZ, C., MCKENNA, A., SHEFLER, E., RAMOS, A. H., STOJANOV, P., CARTER, S. L., VOET, D., CORTES, M. L., AUCLAIR, D., BERGER, M. F., SAKSENA, G., GUIDUCCI, C., ONOFRIO, R. C., PARKIN, M., ROMKES, M., WEISSFELD, J. L., SEETHALA, R. R., WANG, L., RANGEL-ESCARENO, C., FERNANDEZ-LOPEZ, J. C., HIDALGO-MIRANDA, A., MELENDEZ-ZAJGLA, J., WINCKLER, W., ARDLIE, K., GABRIEL, S. B., MEYERSON, M., LANDER, E. S., GETZ, G., GOLUB, T. R., GARRAWAY, L. A. & GRANDIS, J. R. 2011. The Mutational Landscape of Head and Neck Squamous Cell Carcinoma. *Science,* 333**,** 1157-1160.

STRATIGOS, A., GARBE, C., LEBBE, C., MALVEHY, J., DEL MARMOL, V., PEHAMBERGER, H., PERIS, K., BECKER, J. C., ZALAUDEK, I., SAIAG, P., MIDDLETON, M. R., BASTHOLT, L., TESTORI, A., GROB, J. J., EUROPEAN DERMATOLOGY, F., EUROPEAN ASSOCIATION OF, D.-O., EUROPEAN ORGANIZATION FOR, R. & TREATMENT OF, C. 2015. Diagnosis and treatment of invasive squamous cell carcinoma of the skin: European consensus-based interdisciplinary guideline. *Eur J Cancer,* 51**,** 1989-2007.

STRAUB, W. E., WEBER, T. A., SCHAFER, B., CANDI, E., DURST, F., OU, H. D., RAJALINGAM, K., MELINO, G. & DOTSCH, V. 2010. The C-terminus of p63 contains multiple regulatory elements with different functions. *Cell Death Dis,* 1**,** e5.

SU, X., CHAKRAVARTI, D., CHO, M. S., LIU, L., GI, Y. J., LIN, Y. L., LEUNG, M. L., EL-NAGGAR, A., CREIGHTON, C. J., SURAOKAR, M. B., WISTUBA, I. & FLORES, E. R. 2010. TAp63 suppresses metastasis through coordinate regulation of Dicer and miRNAs. *Nature,* 467**,** 986-90.

SU, X., CHAKRAVARTI, D. & FLORES, E. R. 2013. p63 steps into the limelight: crucial roles in the suppression of tumorigenesis and metastasis. *Nat Rev Cancer,* 13**,** 136-43.

SU, X., PARIS, M., GI, Y. J., TSAI, K. Y., CHO, M. S., LIN, Y. L., BIERNASKIE, J. A., SINHA, S., PRIVES, C., PEVNY, L. H., MILLER, F. D. & FLORES, E. R. 2009. TAp63 prevents premature aging by promoting adult stem cell maintenance. *Cell Stem Cell,* 5**,** 64-75.

SULLIVAN, A. & LU, X. 2007. ASPP: a new family of oncogenes and tumour suppressor genes. *Br J Cancer,* 96**,** 196-200.

SULTANA, H., KIGAWA, J., KANAMORI, Y., ITAMOCHI, H., OISHI, T., SATO, S., KAMAZAWA, S., OHWADA, M., SUZUKI, M. & TERAKAWA, N. 2003. Chemosensitivity and p53-Bax pathway-mediated apoptosis in patients with uterine cervical cancer. *Ann Oncol,* 14**,** 214-9.

SUMBUL, A. T., GOGEBAKAN, B., BAYRAM, S., BATMACI, C. Y. & OZTUZCU, S. 2015. MicroRNA 211 expression is upregulated and associated with poor prognosis in colorectal cancer: a case-control study. *Tumour Biol*.

TETZLAFF, M. T., CURRY, J. L., YIN, V., PATTANAPRICHAKUL, P., MANONUKUL, J., UIPRASERTKUL, M., MANYAM, G. C., WANI, K. M., ALDAPE, K., ZHANG, L., PRIETO, V. G. & ESMAELI, B. 2015. Distinct Pathways in the Pathogenesis of Sebaceous Carcinomas Implicated by Differentially Expressed MicroRNAs. *JAMA Ophthalmol*.

THANOS, C. D. & BOWIE, J. U. 1999. p53 Family members p63 and p73 are SAM domain-containing proteins. *Protein Sci,* 8**,** 1708-10.

THOMPSON, S. C., JOLLEY, D. & MARKS, R. 1993. Reduction of solar keratoses by regular sunscreen use. *N Engl J Med,* 329**,** 1147-51.

TOONEN, J., LIANG, L. & SIDJANIN, D. J. 2012. Waved with open eyelids 2 (woe2) is a novel spontaneous mouse mutation in the protein phosphatase 1, regulatory (inhibitor) subunit 13 like (Ppp1r13l) gene. *BMC Genet,* 13**,** 76.

TORDELLA, L., KOCH, S., SALTER, V., PAGOTTO, A., DOONDEEA, J. B., FELLER, S. M., RATNAYAKA, I., ZHONG, S., GOLDIN, R. D., LOZANO, G., MCKEON, F. D., TAVASSOLI, M., FRITZSCHE, F., HUBER, G. F., ROSSLE, M., MOCH, H. & LU, X. 2013. ASPP2 suppresses squamous cell carcinoma via RelA/p65-mediated repression of p63. *Proc Natl Acad Sci U S A,* 110**,** 17969-74.

TRAN, M. N., CHOI, W., WSZOLEK, M. F., NAVAI, N., LEE, I. L., NITTI, G., WEN, S., FLORES, E. R., SIEFKER-RADTKE, A., CZERNIAK, B., DINNEY, C., BARTON, M. & MCCONKEY, D. J. 2013. The p63 protein isoform DeltaNp63alpha inhibits epithelial-mesenchymal transition in human bladder cancer cells: role of MIR-205. *J Biol Chem,* 288**,** 3275-88.

TRIGIANTE, G. & LU, X. 2006. ASPP [corrected] and cancer. *Nat Rev Cancer,* 6**,** 217-26.

TSAI, J. H., DONAHER, J. L., MURPHY, D. A., CHAU, S. & YANG, J. 2012. Spatiotemporal regulation of epithelial-mesenchymal transition is essential for squamous cell carcinoma metastasis. *Cancer Cell,* 22**,** 725-36.

TUCCI, P., AGOSTINI, M., GRESPI, F., MARKERT, E. K., TERRINONI, A., VOUSDEN, K. H., MULLER, P. A., DOTSCH, V., KEHRLOESSER, S., SAYAN, B. S., GIACCONE, G., LOWE, S. W., TAKAHASHI, N., VANDENABEELE, P., KNIGHT, R. A., LEVINE, A. J. & MELINO, G. 2012. Loss of p63 and its microRNA-205 target results in enhanced cell migration and metastasis in prostate cancer. *Proc Natl Acad Sci U S A,* 109**,** 15312-7.

VALOCZI, A., HORNYIK, C., VARGA, N., BURGYAN, J., KAUPPINEN, S. & HAVELDA, Z. 2004. Sensitive and specific detection of microRNAs by northern blot analysis using LNA-modified oligonucleotide probes. *Nucleic Acids Res,* 32**,** e175.

VAN MAANEN, J. M., RETEL, J., DE VRIES, J. & PINEDO, H. M. 1988. Mechanism of action of antitumor drug etoposide: a review. *J Natl Cancer Inst,* 80**,** 1526-33.

VERHOVEN, B., SCHLEGEL, R. A. & WILLIAMSON, P. 1995. Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes. *J Exp Med,* 182**,** 1597-601.

VIVO, M., DI COSTANZO, A., FORTUGNO, P., POLLICE, A., CALABRO, V. & LA MANTIA, G. 2009. Downregulation of DeltaNp63alpha in keratinocytes by p14ARF-mediated SUMO-conjugation and degradation. *Cell Cycle,* 8**,** 3537-43.

VOUSDEN, K. H. & LU, X. 2002. Live or let die: the cell's response to p53. *Nat Rev Cancer,* 2**,** 594-604.

WANG, A., LANDEN, N. X., MEISGEN, F., LOHCHAROENKAL, W., STAHLE, M., SONKOLY, E. & PIVARCSI, A. 2014a. MicroRNA-31 is overexpressed in cutaneous squamous cell carcinoma and regulates cell motility and colony formation ability of tumor cells. *PLoS One,* 9**,** e103206.

WANG, G., CHAN, E. S., KWAN, B. C., LI, P. K., YIP, S. K., SZETO, C. C. & NG, C. F. 2012. Expression of microRNAs in the urine of patients with bladder cancer. *Clin Genitourin Cancer,* 10**,** 106-13.

WANG, L. L., XU, Z., PENG, Y., LI, L. C. & WU, X. L. 2015a. Downregulation of inhibitor of apoptosisstimulating protein of p53 inhibits proliferation and promotes apoptosis of gastric cancer cells. *Mol Med Rep,* 12**,** 1653-8.

WANG, S. H., ZHOU, J. D., HE, Q. Y., YIN, Z. Q., CAO, K. & LUO, C. Q. 2014b. MiR-199a inhibits the ability of proliferation and migration by regulating CD44-Ezrin signaling in cutaneous squamous cell carcinoma cells. *Int J Clin Exp Pathol,* 7**,** 7131-41.

WANG, Y., BU, F., ROYER, C., SERRES, S., LARKIN, J. R., SOTO, M. S., SIBSON, N. R., SALTER, V., FRITZSCHE, F., TURNQUIST, C., KOCH, S., ZAK, J., ZHONG, S., WU, G., LIANG, A., OLOFSEN, P. A., MOCH, H., HANCOCK, D. C., DOWNWARD, J., GOLDIN, R. D., ZHAO, J., TONG, X., GUO, Y. & LU, X. 2014c. ASPP2 controls epithelial plasticity and inhibits metastasis through beta-catenin-dependent regulation of ZEB1. *Nat Cell Biol,* 16**,** 1092-104.

WANG, Y., WANG, L., YIN, C., AN, B., HAO, Y., WEI, T., LI, L. & SONG, G. 2015b. Arsenic trioxide inhibits breast cancer cell growth via microRNA-328/hERG pathway in MCF-7 cells. *Mol Med Rep,* 12**,** 1233-8.

WANG, Y., XU, S. L., WU, Y. Z., ZHAO, M. S., XU, W. J., YANG, H. Y. & LI, Y. X. 2013. Simvastatin induces caspase-dependent apoptosis and activates p53 in OCM-1 cells. *Exp Eye Res,* 113**,** 128-34.

WATT, F. M. 1983. Involucrin and other markers of keratinocyte terminal differentiation. *J Invest Dermatol,* 81**,** 100s-3s.

WESTFALL, M. D., JOYNER, A. S., BARBIERI, C. E., LIVINGSTONE, M. & PIETENPOL, J. A. 2005. Ultraviolet radiation induces phosphorylation and ubiquitin-mediated degradation of DeltaNp63alpha. *Cell Cycle,* 4**,** 710-6.

WILSON, A. M., CHIODO, V. A., BOYE, S. L., BRECHA, N. C., HAUSWIRTH, W. W. & DI POLO, A. 2014. Inhibitor of Apoptosis-Stimulating Protein of p53 (iASPP) Is Required for Neuronal Survival after Axonal Injury. *PLoS One,* 9**,** e94175.

WINER, R. L., HUGHES, J. P., FENG, Q., O'REILLY, S., KIVIAT, N. B., HOLMES, K. K. & KOUTSKY, L. A. 2006. Condom use and the risk of genital human papillomavirus infection in young women. *N Engl J Med,* 354**,** 2645-54.

WRONE, D. A., YOO, S., CHIPPS, L. K. & MOY, R. L. 2004. The expression of p63 in actinic keratoses, seborrheic keratosis, and cutaneous squamous cell carcinomas. *Dermatol Surg,* 30**,** 1299-302.

WU, Z., SUN, L., WANG, H., YAO, J., JIANG, C., XU, W. & YANG, Z. 2012. MiR-328 expression is decreased in high-grade gliomas and is associated with worse survival in primary glioblastoma. *PLoS One,* 7**,** e47270.

XIE, Z. & KLIONSKY, D. J. 2007. Autophagosome formation: core machinery and adaptations. *Nat Cell Biol,* 9**,** 1102-9.

XU, N., ZHANG, L., MEISGEN, F., HARADA, M., HEILBORN, J., HOMEY, B., GRANDER, D., STAHLE, M., SONKOLY, E. & PIVARCSI, A. 2012. MicroRNA-125b down-regulates

matrix metallopeptidase 13 and inhibits cutaneous squamous cell carcinoma cell proliferation, migration, and invasion. *J Biol Chem,* 287**,** 29899-908.

XU, X. T., XU, Q., TONG, J. L., ZHU, M. M., NIE, F., CHEN, X., XIAO, S. D. & RAN, Z. H. 2012. MicroRNA expression profiling identifies miR-328 regulates cancer stem cell-like SP cells in colorectal cancer. *Br J Cancer,* 106**,** 1320-30.

XU, Y., BRENN, T., BROWN, E. R., DOHERTY, V. & MELTON, D. W. 2012. Differential expression of microRNAs during melanoma progression: miR-200c, miR-205 and miR-211 are downregulated in melanoma and act as tumour suppressors. *Br J Cancer,* 106**,** 553-61.

YAMANE, K., JINNIN, M., ETOH, T., KOBAYASHI, Y., SHIMOZONO, N., FUKUSHIMA, S., MASUGUCHI, S., MARUO, K., INOUE, Y., ISHIHARA, T., AOI, J., OIKE, Y. & IHN, H. 2013. Down-regulation of miR-124/-214 in cutaneous squamous cell carcinoma mediates abnormal cell proliferation via the induction of ERK. *J Mol Med (Berl),* 91**,** 69-81.

YANG, A., KAGHAD, M., WANG, Y., GILLETT, E., FLEMING, M. D., DOTSCH, V., ANDREWS, N. C., CAPUT, D. & MCKEON, F. 1998. p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol Cell,* 2**,** 305-16.

YANG, J. P., HORI, M., SANDA, T. & OKAMOTO, T. 1999. Identification of a novel inhibitor of nuclear factor-kappaB, RelA-associated inhibitor. *J Biol Chem,* 274**,** 15662-70.

YANG, S. F., LEE, W. J., TAN, P., TANG, C. H., HSIAO, M., HSIEH, F. K. & CHIEN, M. H. 2015. Upregulation of miR-328 and inhibition of CREB-DNA-binding activity are critical for resveratrol-mediated suppression of matrix metalloproteinase-2 and subsequent metastatic ability in human osteosarcomas. *Oncotarget,* 6**,** 2736-53.

YI, R., POY, M. N., STOFFEL, M. & FUCHS, E. 2008. A skin microRNA promotes differentiation by repressing 'stemness'. *Nature,* 452**,** 225-9.

YOON, J. H., LEE, C. S., O'CONNOR, T. R., YASUI, A. & PFEIFER, G. P. 2000. The DNA damage spectrum produced by simulated sunlight. *J Mol Biol,* 299**,** 681-93.

YUAN, J., ZHENG, Z., ZHENG, Y., LU, X., XU, L. & LIN, L. 2015. microRNA-328 is a favorable prognostic marker in human glioma via suppressing invasive and proliferative phenotypes of malignant cells. *Int J Neurosci*.

ZANGEN, R., RATOVITSKI, E. & SIDRANSKY, D. 2005. Delta Np63 alpha levels correlate with clinical tumor response to cisplatin. *Cell Cycle,* 4**,** 1313-1315.

ZHANG, B., XIAO, H. J., CHEN, J., TAO, X. & CAI, L. H. 2011. Inhibitory member of the apoptosis-stimulating protein of p53 (ASPP) family promotes growth and tumorigenesis in human p53-deficient prostate cancer cells. *Prostate Cancer Prostatic Dis,* 14**,** 219-24.

ZHANG, P., WANG, L., RODRIGUEZ-AGUAYO, C., YUAN, Y., DEBEB, B. G., CHEN, D., SUN, Y., YOU, M. J., LIU, Y., DEAN, D. C., WOODWARD, W. A., LIANG, H., YANG, X., LOPEZ-BERESTEIN, G., SOOD, A. K., HU, Y., ANG, K. K., CHEN, J. & MA, L. 2014. miR-205 acts as a tumour radiosensitizer by targeting ZEB1 and Ubc13. *Nat Commun,* 5**,** 5671.

ZHANG, X., WANG, M., ZHOU, C., CHEN, S. & WANG, J. 2005. The expression of iASPP in acute leukemias. *Leuk Res,* 29**,** 179-83.

ZHAO, W. H., WU, S. Q. & ZHANG, Y. D. 2013. Downregulation of miR-124 promotes the growth and invasiveness of glioblastoma cells involving upregulation of PPP1R13L. *Int J Mol Med,* 32**,** 101-7.

ZHOU, J., LIU, R., LUO, C., ZHOU, X., XIA, K., CHEN, X., ZHOU, M., ZOU, Q., CAO, P. & CAO, K. 2014. MiR-20a inhibits cutaneous squamous cell carcinoma metastasis and proliferation by directly targeting LIMK1. *Cancer Biol Ther,* 15**,** 1340-9.

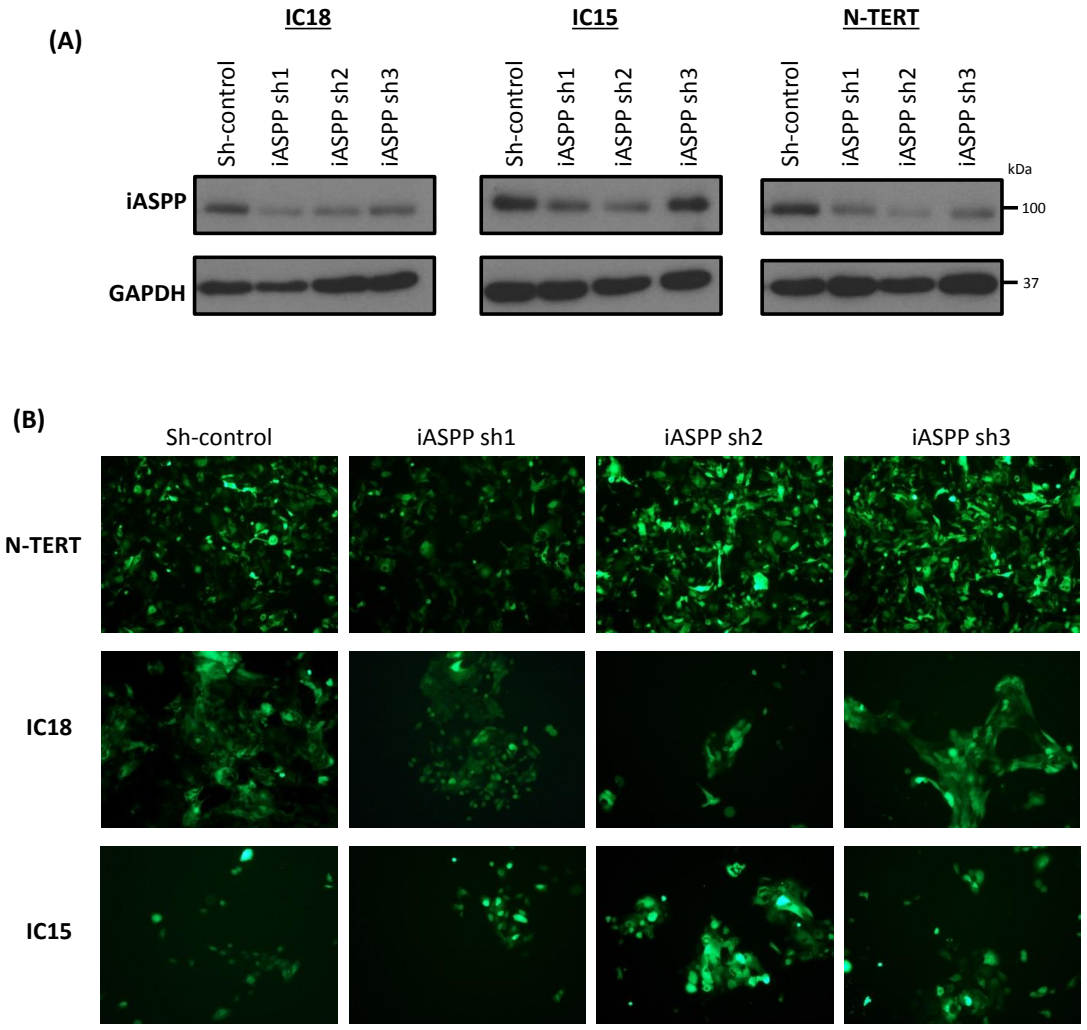
ZHOU, L., WANG, Y., OU, C., LIN, Z., WANG, J., LIU, H., ZHOU, M. & DING, Z. 2015. microRNA-365-targeted nuclear factor I/B transcriptionally represses cyclin-dependent kinase

6 and 4 to inhibit the progression of cutaneous squamous cell carcinoma. *Int J Biochem Cell Biol,* 65**,** 182-91.

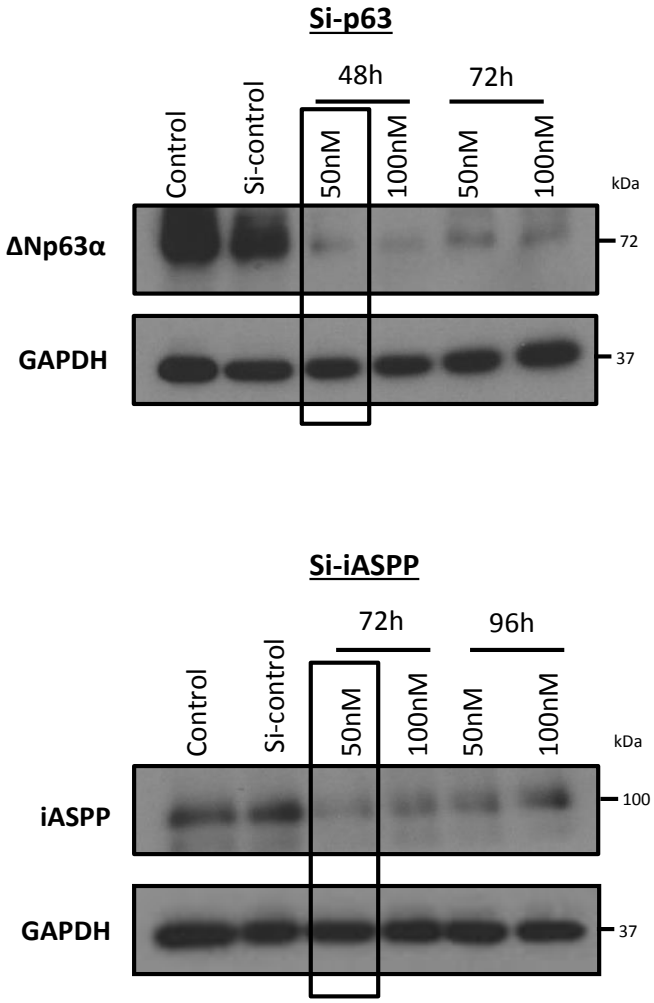
ZHOU, M., LIU, W., MA, S., CAO, H., PENG, X., GUO, L., ZHOU, X., ZHENG, L., GUO, L., WAN, M., SHI, W., HE, Y., LU, C., JIANG, L., OU, C., GUO, Y. & DING, Z. 2013. A novel onco-miR-365 induces cutaneous squamous cell carcinoma. *Carcinogenesis,* 34**,** 1653-9.

ZIEGLER, A., JONASON, A. S., LEFFELL, D. J., SIMON, J. A., SHARMA, H. W., KIMMELMAN, J., REMINGTON, L., JACKS, T. & BRASH, D. E. 1994. Sunburn and p53 in the onset of skin cancer. *Nature,* 372**,** 773-6.

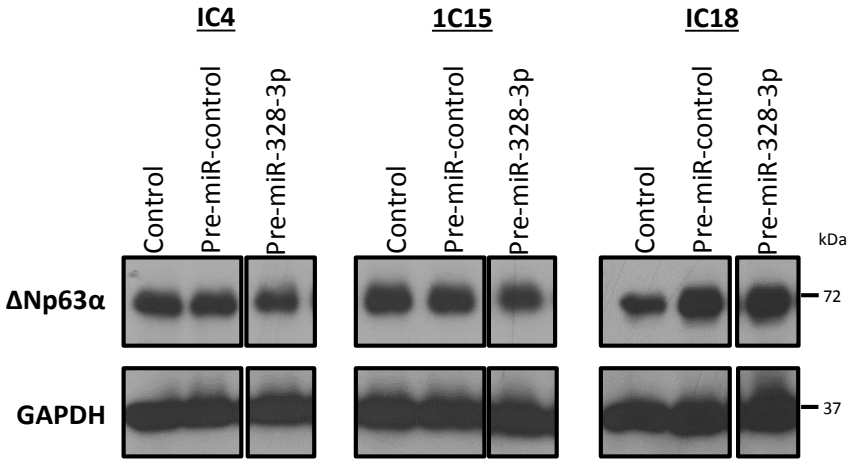
Chapter 8: Appendix



Supplementary figure 4.1. ShRNA in cSCC and N-TERT cell lines. (A) cSCC and N-TERT cells were either treated with a non-targeting control (sh-control), silenced for iASPP using individual shRNA (sh-1, sh-2, sh-3). Protein was extracted and analysed by western blotting for iASPP knockdown. GAPDH was used as a loading control. **(B)** Immunofluorescence images of cells infected with GFP-tagged shRNA - sh-control, sh-1, sh-2, sh-3.



Supplementary figure 4.2. SiRNA optimisation. cSCC cell line IC15 treated with 50nM and 100nM siRNA for 48 and 72h (si-p63) or siRNA for 72 and 96h. GAPDH was used as a loading control.



Supplementary figure 4.3. Mir-328-3p overexpression is unable to affect p63 expression. Cells either untreated (control), treated with a Pre-miR control or with Pre-miR-328-3p. Lysates were run on western blot and ΔNp63α protein expression was determined. GAPDH was used as a loading control**.**